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CLINICAL AND EXPERIMENTAL

HEART DISEASE AND LIVER FUNCTION*

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HERETOFORE, studies of the relationship of hepatic function to heart disease have been based solely upon clinical observations.[1-5] A review of the literature fails to disclose any studies correlating liver function and cardiac disease, as determined by precise methods. In the present report we have attempted to study this relationship by means of the bromsulfalein retention test and by the measurement of the velocity of blood flow and the venous pressure.

MATERIAL AND METHODS

A group of 59 patients, free from hepatobiliary disease was studied. There were 29 men and 30 women, ranging in age from 19 to 79 years. These patients were studied clinically and quantitatively as to the status of the circulation and liver. For the purpose of this investigation the patients were divided as follows:

A. *Control Group:* Patients free from any demonstrable evidence of heart disease. Five patients with uncomplicated hyperthyroidism were included in this group.

B. *Cardiac Group:* These patients, evidencing definite heart disease, were for practical purposes[6] subdivided on a functional basis as follows:

1. Fully compensated heart disease.

2. Mild cardiac decompensation, evidenced by exertional dyspnea and cyanosis.

3. Moderate cardiac decompensation, evidenced by dyspnea, cyanosis, and pulmonary congestion, the last named being demonstrable clinically or by roentgen-ray examination.

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4. Marked cardiac decompensation, evidenced by symptoms and signs of groups 2 and 3 plus hepatomegaly, edema, or ascites. Three patients were listed in more than one group in accordance with their changing functional cardiac status.

In the group of cardiac patients were 14 with hypertensive cardiovascular disease, 16 with arteriosclerotic heart disease, 5 with myocardial infarction, 6 with rheumatic heart disease, 2 with subacute bacterial endocarditis, and one with adhesive pericarditis.

The circulation was studied quantitatively by means of the venous pressure and the magnesium sulfate and ether circulation time tests. The venous pressure was measured directly by the use of an L-tube, calibrated in millimeters and centimeters, to which was attached an 18 gauge needle. Both tube and needle were thoroughly flushed with 2.5 per cent sodium citrate solution. The patient was in recumbent position, except when prevented by extreme cardiac insufficiency. The arm was abducted to a 45 degree angle and supported on a pillow which was so arranged as to place the antecubital fossa 5 cm. posterior to the level of the fourth sternochondral junction. A tourniquet was applied to the arm, and the needle was inserted into a large antecubital vein. The patient was cautioned to lie and breathe as quietly as possible. After the fluctuations of the venous column of blood subsided, the pressure was read directly in centimeters of blood. With the needle left in situ 5 c.c. of warm 10 per cent magnesium sulfate solution were injected as rapidly as possible and the end point was determined, giving the arm-to-tongue circulation time. One minute later 5 minims of ether mixed with 5 minims of normal saline solution were injected and the end point was determined, giving the arm-to-lung circulation time.

By means of the bromsulfalein retention test an index of the liver function was obtained. A dose of 5 mg. of bromsulfalein per kilogram of body weight was employed. The bromsulfalein determination followed or preceded the study of the circulation as closely as possible, usually within one or two hours. After injection of the dye, readings were made from specimens of blood collected at intervals of five, fifteen, and thirty minutes.

A total of 70 sets of circulation times, venous pressure, and bromsulfalein determinations were performed in the 59 patients.

Criteria.—The following values were used as normals in determining the status of the circulation:

Venous pressure: 3 to 12 cm. of blood.

Arm-to-tongue circulation time (magnesium sulfate circulation time[6]) eight to fourteen seconds.

Arm-to-lung circulation time (ether circulation time) three to eight seconds.

Lung-to-tongue circulation time (magnesium sulfate circulation time minus ether circulation time) up to eight seconds.

Failure of the left side of the heart was evidenced by a reading of lung-to-tongue circulation time greater than eight seconds. Failure of the right side of the heart was evidenced by a reading of ether circulation time greater than eight seconds and/or by a reading of the venous pressure over 12 cm. of blood.

Bromsulfalein retention of 10 per cent was considered as the upper limits of normal.

RESULTS

A. *Control Group.*—Fifteen sets of determinations were performed on 15 noncardiac patients. In 14 patients the bromsulfalein liver function test was normal, whereas one patient showed an abnormal bromsulfalein retention without demonstrable cause.

B. *Cardiac Group.*—In 10 patients comprising group 1 (fully compensated heart disease), 12 of 13 determinations made showed agreement and one determination showed disagreement between the cardiac status and the liver function. Group 2, consisting of 10 patients, disclosed 11 determinations in which there was agreement and 3 with disagreement. Of 14 determinations made on 14 patients in group 3, 6 determinations showed the cardiac status and the liver function tests to be in agreement and 8 showed disagreement. In group 4, consisting of 13 patients, 11 of 14 determinations showed agreement between the cardiac status and liver function, whereas 3 showed disagreement.

Three cases are worthy of special mention. One patient, a 63-year-old man with acute myocardial infarction, at first disclosed failure of the right and left sides of the heart, as evidenced by the circulation time. There was marked cyanosis which persisted throughout a prolonged convalescence. Although the magnesium sulfate circulation time decreased steadily from 25.4 seconds to 14.4 seconds within a period of twenty-two days, with a corresponding drop in ether circulation time from 16.0 to 7.6 seconds, the parallel bromsulfalein determination ranged from 40 per cent to 28 per cent retention. It is very probable that the anoxemia, as evidenced by the cyanosis, may have been a causal factor in the continuance of the abnormal bromsulfalein retention. Another patient, a 60-year-old woman with decompensation due to hypertensive cardiovascular disease, showed exceptional parallelism between the liver function and the cardiac status as the state of compensation progressively improved, both clinically and by the method of measurement described above. A third patient, a 52-year-old woman with rheumatic aortic stenosis complicated by hyperthyroidism, showed slight abnormal bromsulfalein retention (12 per cent) with moderate failure of the right side of the heart; venous pressure 14.8 cm.; arm-to-tongue (magnesium sulfate) circulation time 15.8 seconds; and arm-to-lung (ether) circulation time 10.6 seconds. It is to be noted in the foregoing case that the circulation times would undoubtedly have been much higher were it not for the marked tendency of the hyperthyroidism to decrease the circulation time.

DISCUSSION

Our studies indicate that the bromsulfalein excretion is normal in the absence of heart failure (control and compensated heart disease groups). In mild degrees of cardiac failure (group 2) the bromsulfalein excretion is impaired in close parallelism with the status of the circulation. Incidentally, it is worthy of mention that in mild degrees of cardiac decompensation the circulation times and venous pressure may fall within normal limits in a fairly high percentage of patients, a fact previously brought out by the authors.[6]

It is noteworthy that in patients with definite pulmonary congestion (group 3) the correlation of hepatic function with the condition of the circula-

TABLE I

CLINICAL CLASSIFICATION OF CASES	NUMBER OF PATIENTS	NUMBER OF TESTS	A. BROMSULFALEIN EXCRETION	B. CARDIAC STATUS AS DETERMINED BY CIRCULATION TIMES AND VENOUS PRESSURE				CORRELATION OF TESTS‡ A AND B			
								AGREEMENT		DISAGREEMENT	
				NO HEART FAILURE	HEART FAILURE PRESENT			NUMBER	%	NUMBER	%
					RIGHT	LEFT	BOTH				
Controls*	15	15	Normal	14	0	0	0	14	93.5	1	6.5
			Abnormal	1	0	0	0				
1. Compensated heart disease	10	13	Normal	12	0	0	0	12	92.3	1	7.7
			Abnormal	1	0	0	0				
2. Mild cardiac failure	10	14	Normal	6	1	0	0	11	78.5	3	21.5
			Abnormal	2	1	2	2				
3. Moderate cardiac failure†	14	14	Normal	0	1	5	2	6	42.8	8	57.2
			Abnormal	0	1	5	0				
4. Severe cardiac failure	13	14	Normal	0	1	1	1	11	78.5	3	21.5
			Abnormal	0	0	5	6				

*Includes 5 cases of hyperthyroidism uncomplicated by cardiac disease.

†Includes one case of hyperthyroidism.

‡A test consists of bromsulfalein determination and circulation times and venous pressure.

§There were 59 patients of whom 3 were listed in more than one group in accordance with their changing functional cardiac status.

tion is frequently paradoxical. A study of Table I indicates 8 patients with cardiac failure with normal bromsulfalein excretion. On the other hand, 6 additional patients in this group show impaired bromsulfalein excretion. Of the 14 cases, 4 presented failure of the right side of the heart, either alone or combined with failure of the left side of the heart. Yet, only one of these 4 cases showed increased bromsulfalein retention. Consequently, it is probable that in this group (group 3) the factor of increased venous pressure, producing increased mechanical intrahepatic pressure, is apparently of little causative importance in the impairment of the excretion of bromsulfalein. On the other hand, 7 patients with failure of the left side of the heart, either alone or combined with failure of the right side of the heart, presented normal bromsulfalein excretion and 5 patients with failure of the left side of the heart presented impaired bromsulfalein excretion. Apparently the only factor that may be responsible for impaired bromsulfalein excretion in these cases is the increased degree of oxygen unsaturation of the arterial blood. The liver depends on the blood supplied to it by the hepatic artery for its oxygen, although only 25 per cent of the organ's total blood supply is derived from this source.[7] It would seem, therefore, that anoxemia is the factor determining the development of impaired bromsulfalein excretion in cardiac decompensation of this degree.

In the 14 determinations in cases with severe cardiac failure (group 4) there were 8 cases of predominant failure of the right side of the heart, either alone or combined with failure of the left side of the heart. Of these, 6 presented impaired bromsulfalein excretion. In 6 patients with failure of the left side of the heart, 5 showed impaired bromsulfalein excretion. Therefore, it is reasonable to conclude that in severe cardiac failure of this degree, bromsulfalein excretion is usually impaired.

Although there is a tendency to parallelism between the bromsulfalein excretion and the degree of cardiac failure, we were unable to determine any mathematical correlation between these two factors.

SUMMARY AND CONCLUSIONS

1. The bromsulfalein retention test was correlated with the venous pressure and the blood velocity in 59 patients.

2. A total of 70 sets of magnesium sulfate (arm-to-tongue) circulation times, ether (arm-to-lung) circulation times, venous pressure, and bromsulfalein determinations were made.

3. In general, the bromsulfalein excretion is normal in the absence of heart failure. In mild degree of heart failure the bromsulfalein excretion closely parallels the status of the circulation. The relationship of hepatic function with the condition of the circulation is frequently paradoxical in moderate degree of cardiac failure associated with definite pulmonary congestion. Bromsulfalein excretion is nearly always impaired in severe cardiac failure.

4. There is a tendency to parallelism between the bromsulfalein excretion and the degree of cardiac failure, although this relationship is not mathematical.

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A SIMPLIFIED BEDSIDE TEST FOR LATENT JAUNDICE*

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AN ORDINARY, thin, 3 inch rubber band is the only apparatus required for this test, the technique of which is as follows: The rubber band is placed about the patient's upper arm, drawn taut mesially away from the skin, and then released. A typical "triple response" of Lewis[1] then takes place. We are not concerned with either the red reaction or the flare. A wheal (third phase of the "triple response") appears in five to fifteen minutes. The surrounding skin is then stretched in order to blanch the area, which is the site of the erythematous flare. This may be done advantageously by pressure over a glass slide. The colors of the wheal and of the surrounding skin are compared in either day or artificial light, the former being preferred. A positive test for jaundice is characterized by yellow coloration of the wheal.

The physiologic principles underlying the test have been well known for considerable time. In 1931 Klein[2] demonstrated that in cases of jaundice wheals produced by the intracutaneous injection of histamine were darker yellow than the surrounding skin. He further stated that when the bilirubin content of the blood was raised to levels insufficient to produce clinically detectable jaundice, histamine wheals showed yellow coloration. In 1936, Brodribb and Cullinan[3] reported quantitative studies of this method. They injected 0.1 mg. of histamine in 1 minim of sterile solution intracutaneously in the upper arm or back. They were able to detect a yellow coloration of the wheal in cases of biliary obstruction or hepatic damage when the blood bilirubin was 0.5 mg. or more per 100 c.c. In cases of hemolytic jaundice positive tests were not seen unless the blood bilirubin concentration was at least 1.1 to 1.4 mg. per 100 c.c. These authors mentioned the possibility of positive tests in carotinemia and allied diseases in which there is increased pigment in the blood.

The wheal of the "triple response" of Lewis, which is due perhaps to liberated histamine or "H-substance," is physiologically indistinguishable from

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wheals produced by histamine injected intracutaneously. Any brisk superficial localized trauma may be substituted for the rubber band method of raising the wheal, but because of its simplicity and convenience, this latter procedure is specifically recommended.

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OBSERVATIONS ON ADDITIONAL INSTANCES OF A SUPERNORMAL PHASE IN THE HUMAN HEART*

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IN 1924 Lewis and Master[1] reported a case of heart block in which complete dissociation of the auricles and ventricles was interrupted by occasional conducted beats. It was noted that in order for conduction from auricle to ventricle to occur, the P wave had to fall in a definite time period after the preceding QRS complex of the idioventricular rhythm. Cases of this type are not rare, and an example (Case 1) has been chosen from two which I have encountered.

REPORT OF CASES

CASE 1.—The patient was a man 73 years of age who suffered from attacks of syncope. Except for bradycardia and generalized senescent changes, physical examination gave negative results. An electrocardiogram (Fig. 1) revealed an idioventricular rhythm with a cycle of two seconds, and occasional interruption or interference of the cycle by premature ventricular complexes which followed a P wave with a constant P-R interval. When the P waves which were followed by ventricular complexes were charted (Fig. 2), it was seen that they fell between 0.56 and 0.88 second after onset of the preceding QRS complex; P waves falling outside this phase were not conducted.

Comment.—Lewis and Master explained this phenomenon by the presence of a supernormal phase of conductivity similar to a biologic phenomenon observed by Adrian and Lucas[2] in 1912 and described in greater detail by Adrian[3] in 1921. These observers demonstrated that in injured, excitable tissue there was a short period during recovery from a previous stimulus in which the tissue became hypersensitive to new stimuli. In 1925 Ashman[4] observed the existence of the supernormal phase of conductivity in the hearts of turtles, in which varying degrees of auriculoventricular block had been produced. The work of Hoff and Nahum[5] in 1938 indicated that a supernormal phase of excitability could be present in relatively normal mammalian ventricular muscle.

A second type of cardiac mechanism exhibiting evidence of the supernormal phase is much more rare and is that of paroxysmal heart block occurring con-

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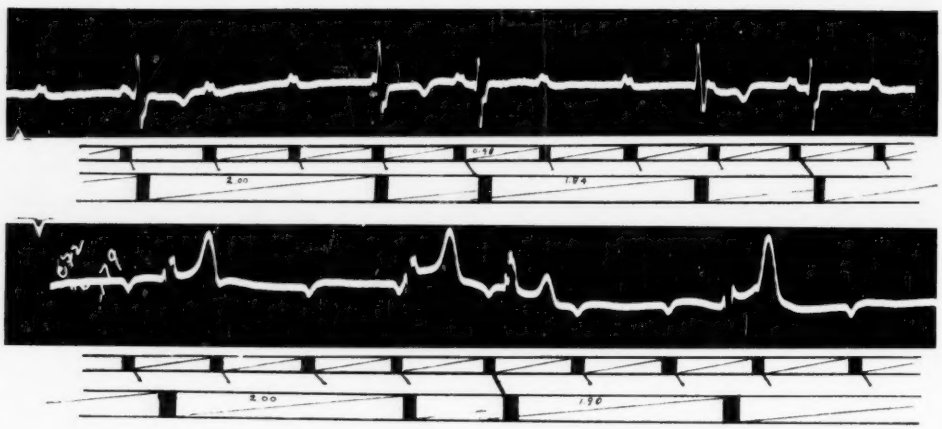

Fig. 1.—Complete heart block except for occasional auriculoventricular conduction. Two conducted beats are shown in the upper tracing (Lead II) and one in the lower tracing (Lead CF_2). The ventricular complex of the conducted beat has a different shape from the QRS complex of the idioventricular rhythm. It is also interesting to note that the returning cycle of idioventricular rhythm is shorter than might be expected.

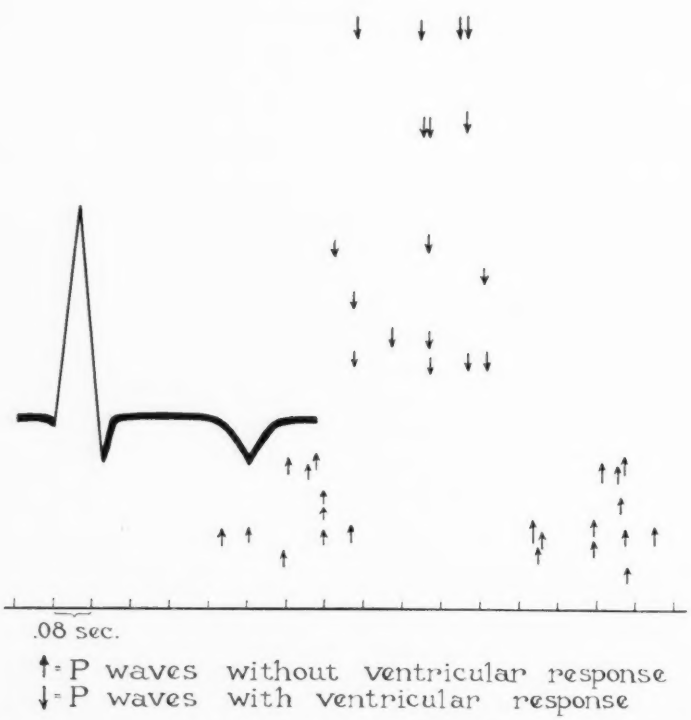

Fig. 2.—The positions of all the P waves that were followed by auriculoventricular conduction are compared with the positions of P waves associated with auriculoventricular block. There is a slight overlapping of two groups at the beginning of the time phase of favored conductivity, but not at the end of this phase. The P-R interval of the conducted beats is not charted but was normal and the same for all conducted beats.

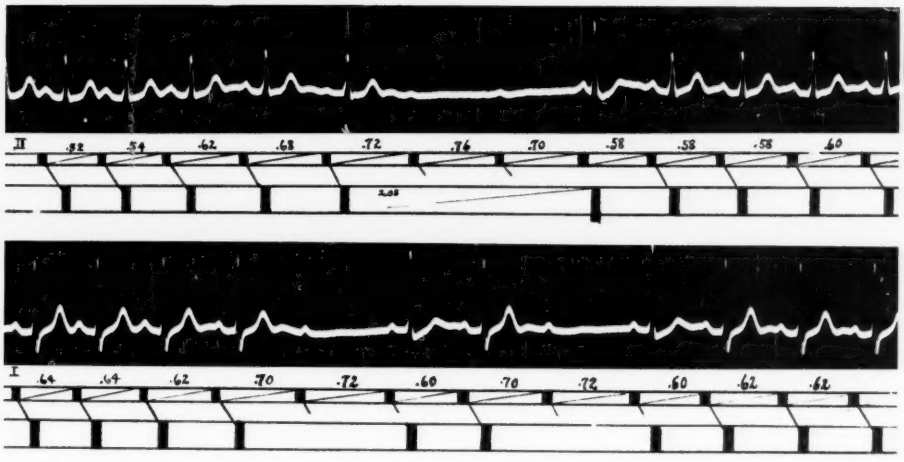

Fig. 3.—Paroxysms of complete auriculoventricular block following auricular slowing and resumption of auriculoventricular conductivity only when a P wave has fallen in a definite time relationship after an idioventricular beat are demonstrated. The phenomenon may be seen once in the upper tracing and twice in the lower. The P-P intervals are given in hundredths of seconds.

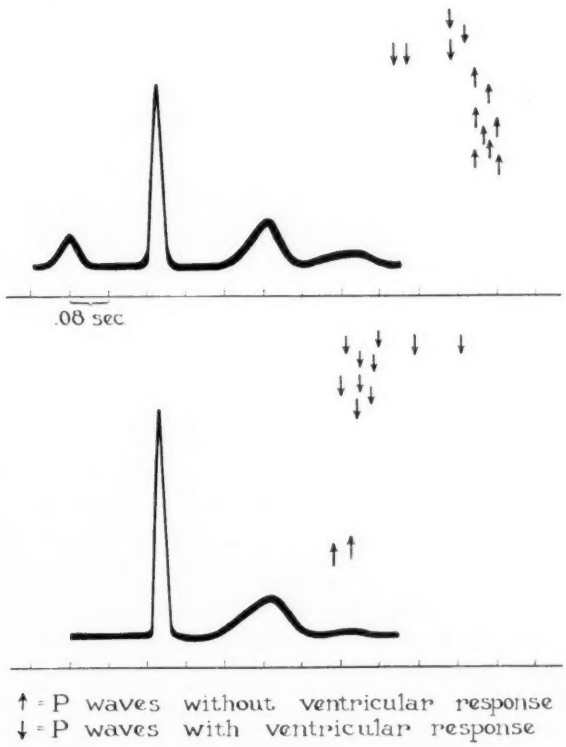

Fig. 4.—Upper portion demonstrates the position of P waves, always followed by auriculoventricular conduction and the maintenance of normal rhythm, and the position of P waves followed by auriculoventricular block. In the original tracing numerous P waves were present, but as they all had the same grouping, many were omitted in this figure as their numbers added nothing to a smaller representative group. In the lower portion of the chart P waves which followed an idioventricular beat and were associated with auriculoventricular conduction and the resumption of normal rhythm may be seen. Except for one possible exception P waves outside this time relationship were not associated with auriculoventricular conduction.

comitantly with auricular slowing. The electrocardiographic records obtained in Case 2 are similar to those obtained in a case reported by Wilson and Herrmann[6] and described later in detail by Ashman and Herrmann,[7] and to those in a case reported by Kline, Conn, and Rosenbaum.[8]

CASE 2.—The patient, a man 29 years of age, registered at the Mayo Clinic in February, 1940, and related a history of frequent attacks of syncope of two years' duration. It was noted on physical examination that the fainting spells were associated with ventricular asystole and that attacks could be reproduced by pressure on either of the carotid sinuses. There was a rather distant, harsh, aortic systolic murmur, and the heart was slightly enlarged by roentgenographic examination. History of rheumatic fever was not obtained.

On electrocardiographic study it was noted that complete auriculoventricular dissociation was dependent on auricular slowing. Auriculoventricular conduction was never re-established until one or more idioventricular beats had occurred, and the P wave of the first beat to be conducted had to have a definite time relationship to the preceding QRS complex. The tracings reproduced in Fig. 3 are representative of numerous records obtained, both in spontaneous attacks of auriculoventricular dissociation and in attacks induced by pressure on a carotid sinus. In the upper tracing reproduced in Fig. 3 it may be seen that there is a gradual slowing of the auricular rate, the cycle increasing from 0.52 to 0.76 second, with a gradual displacement of the P wave away from the preceding complex. It was noted that as soon as the P wave falls away from the U wave phase of the tracing, conduction to the ventricle no longer occurred. In the lower tracing of the same figure the same phenomenon occurs twice. It may be pointed out again that there was no resumption of the normal rhythm until an idioventricular beat has occurred, that is, an idioventricular beat had to sensitize the conducting tissues from below before an auricular impulse could be conducted from above. When the P waves were charted (Fig. 4), it was found that when they fell more than 0.68 second after the preceding QRS complex auriculoventricular dissociation occurred. With return of auriculoventricular conduction the P waves fell between 0.40 and 0.65 second after the preceding QRS complex except for the one exception charted. That a vagal influence on the conduction mechanism is unlikely, is indicated by the fact that temporary resumption of conduction may occur while the auricular rate is still slow. An explanation dependent on an increased blood supply to the bundle following a period of block does not prove satisfactory, since the time of conducted beats is independent of the periods of ventricular standstill. The outstanding difference between this case and the usual case of Adams-Stokes' attacks dependent on paroxysmal heart block is that in the latter, heart block usually is preceded by an increase in the auricular rate and failure of recovery in the conducting tissues.

Since the patient had frequent attacks of syncope, digitalis was administered in the hope that it might produce permanent auriculoventricular block and relieve the symptoms. It was found, however, that digitalis caused complete block only when symptoms of mild intoxication from digitalis were present. Administration of digitalis, therefore, was discontinued. After the patient returned home, the symptoms soon disappeared entirely, and he was able to do his routine work on the farm.

The patient returned to the clinic two years later, in February, 1942. It was found that he had complete auriculoventricular dissociation with a ventricular rate of 40. Subcutaneous administration of 1 c.c. of epinephrine (1:1,000) or $\frac{1}{150}$ grain (0.0013 Gm.) of atropine did not have any effect in changing the complete block. His general condition at this time was excellent, although the aortic systolic murmur was louder, a faint diastolic murmur was present at the left of the sternum and the heart had increased slightly in size. During a continued period of auscultation the first heart sound occasionally had the accentuated loudness characteristic in certain cases of complete heart block.

SUMMARY

The presence of the supernormal period in the human heart has been used to explain cases of interference dissociation in which the auricles are beating

more rapidly than the ventricles, cases of paroxysmal heart block in which cessation and resumption of auriculoventricular conduction have a phasic dependency on the immediately preceding electrical events and cases of possible parasystolic rhythm. An example is given of each of the first two types of mechanism which are best explained by the existence of a supernormal period in excitability of the conduction tissues. Case 2 is considered of some importance, as it is another instance of the undoubtedly rare cases in which the supernormal period has played a role in the maintenance of normal sinus rhythm. Following establishment of complete block, the patient has remained in relatively good health for two years.

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PERCUTANEOUS TYPHOID PROPHYLAXIS*

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PERIODIC typhoid prophylaxis is necessary if there is continuous possibility of contact with the typhoid bacillus. Reinoculation by the intradermal route presents a technique that has promise of being the method of choice.[1] Since large numbers of persons are sometimes involved, it was deemed advisable to study the percutaneous route of administering antigenic stimulation in an attempt to simplify the procedure further.

It has been shown that many diverse substances are absorbed through the skin.[2, 3] In previous investigations huge doses of bacilli were utilized—as much as 100 mg. of bacilli by weight with an emulsion base.[4] Since such enormous amounts of antigen would be impossible of production, doses were used which seemed likely to be both antigenic and feasible of manufacture.

The typhoid strain utilized was isolated in 1926 at the Cincinnati General Hospital. Its Vi content is unknown. Standard vaccine was prepared, two billion bacilli to the cubic centimeter. The production of "H" antibodies was studied, since a measure of protective antibodies was not possible; and the production of "O" antibodies following intradermal inoculation was found to be

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TABLE I

GROUP	PATIENT	INITIAL TITER PRIMARY INOCULATION	RESPONSE TO PRIMARY STIMULATION SECOND STIMULATION	RESPONSE TO SECOND STIMULATION (INTRACUTANEOUS)	RESPONSE TO SECOND STIMULATION (PERCUTANEOUS)	INITIAL TITER PERCUTANEOUS RESTIMULATION	RESPONSE TO RESTIMULATION
		2/23/41	3/6/41	3/15/41	3/17/41	8/9/41	8/25/41
A (Control)	1	0	20	20		–	–
	2	0	320	640		40	40
	3	0	320	320		–	–
	4	0	320	640		–	–
	5	0	160	320		–	–
	6	0	320	640		0	20
	7	10	320	640		–	–
B (Disintegrated bacilli)	1	0	160		40	–	–
	2	0	40		10	0	0
	3	0	1,280		1,280	80	80
	4	10	40		40	20	20
	5	0	40		40	10	40
	6	10	10		10	0	0
	7	0	160		320	0	0
	8	0	40		20	–	–
	9	0	1,280		1,280	0	–
C (Whole bacilli)	1	0	640		320	–	–
	2	0	160		320	10	80
	3	0	160		80	0	0
	4	40	1,280		1,280	–	–
	5	0	40		40	–	–
	6	0	80		40	20	40
	7	0	40		10	–	–
	8	0	20		20	0	0
Total	24	24	24	7	17	13	13

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practically nil by Van Gelder.[5] Our titration of "O" antibody gave the same result. Our first attempt at percutaneous stimulation utilized 20 billion typhoid bacilli a day for three consecutive days; the second employed a single dose of 200 billion bacilli.

Twenty-four patients of the Hamilton County Chronic Disease Hospital, who had no history of either typhoid fever or prophylaxis, were divided into three groups. Initially each patient received an intracutaneous primary antigenic stimulation of 0.1 c.c. of the prepared vaccine. The control group, A, was reinoculated fourteen days later with 0.1 c.c. intracutaneously. Group B at the same time vigorously massaged an emulsion of 20 billion disintegrated (alternate freezing and melting) bacilli into the skin of the forearm, and group C an equal dose of whole bacilli. The emulsions were applied to alternate forearms for three consecutive days. The base was Jergens lotion, and each 0.2 c.c. contained the required dose.

Nine days after the termination of the secondary antigenic stimulations, blood sera titrations revealed equivocal results in groups B and C, and increases in 5 of 7 persons in group A. Six months later 13 patients were retested, 2 from group A, 7 from group B, and 4 from group C. (The other patients were not available.) The initial serum titrations showed marked losses in all. Each patient vigorously massaged an emulsion of olive oil and whole bacilli into the forearm; each 0.2 c.c. dose contained 200 billion bacilli. Sixteen days later serum titrations revealed no significant increases in titer.

With the technique employed, no appreciable typhoid "H" antibody was stimulated by percutaneous vaccination.

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ACQUIRED DRUG RESISTANCE IN THE HEMOLYTIC STREPTOCOCCUS*

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MAC LEOD and Daddi,[1] and subsequently Lowell and co-workers[2] showed that some strains of pneumococci, previously susceptible to the action of sulfapyridine, can be rendered "resistant" by growing them in gradually increasing concentrations of the drug in vitro. Recently Hadley and Hadley[3] reported somewhat similar results in the case of a group A hemolytic streptococcus. Using increasing concentrations of sulfanilamide and an incubator temperature of 40° C., they were able to show an "adaptation" of the organism from 2.5 to 10 mg. per 100 c.c. drug concentration. They also noted a change from the mucoid to the smooth phase coincidentally with this adaptation. The present work was planned to investigate the production of drug resistance in vitro in the case of the hemolytic streptococcus, the association of phase transformations, and the duration and stability of the resulting modified strains.

The organism used throughout these experiments was a group A hemolytic streptococcus, freshly isolated from a case of acute pharyngitis. Culture media included plain blood agar plates without added peptone and a meat infusion broth of pH 6.8 likewise without added peptone. At one point in the experiments 20 per cent ascitic fluid was added to the standard broth to produce an enriched medium. The varying concentrations of the drug were obtained by adding the proper amounts of stock solutions of 50 or 100 mg. per 100 c.c. of sulfanilamide in broth.

In describing the various phases of the strains studied we have followed closely the outline of Dawson, Hobby, and Olmstead.[4]

At the outset considerable difficulty was encountered in selecting a suitable organism, for most strains whether freshly isolated or from stock cultures were little affected by concentrations up to 50 mg. per 100 c.c. of sulfanilamide in broth even when a small amount of inoculum was used. Finally the one used, GA, was found on repeated tests to show no growth in concentrations above 7.5 mg. per 100 c.c. sulfanilamide in broth. Morphologically, this organism closely resembled the other strains previously discarded. It formed small rounded colonies on blood agar whitish to reflected light with milky centers and no mucoid border and with a smooth margin when examined under low power. It formed homogeneous suspensions in saline and was not virulent for mice. The lethal dose was between 1 and 2 ml. of a twenty-four-hour broth culture. No capsules could be demonstrated. It resembled the typical "smooth" colony described by other workers. During the course of subsequent experiments it remained essentially stable, and only during the latter

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half did it show some tendency toward the M-S form, as evidenced by slightly increased size and flatness of the colony on blood agar, and by the occasional development of a mucoid "fringe."

Over a period of two weeks GA was subjected to growth in increasing concentrations of sulfanilamide in broth at a temperature of 37.5° C. This was begun at a concentration of 2 mg. per 100 c.c. and carried up by daily subcultures to a concentration of 100 mg. per 100 c.c. The resulting strain labeled GAR after being grown for several transfers in plain broth was tested for drug susceptibility at the same time as the parent strain GA which had meanwhile been carried in plain broth. The inoculum was small, i.e., one standard loopful of a twenty-four-hour broth culture was mixed with 5 c.c. of broth and a loopful of this mixture was used for the test. The results appear in Table I.

TABLE I

INITIAL COMPARISON OF DRUG RESISTANT AND ORIGINAL STRAIN

DRUG CONCENTRATION		0	5	7.5	10	15	20	25	35	50	75	100
GA	Broth	##	#	0	0	0	0	0	0	0	0	0
	Agar*	##	#	0	0	0	0	0	0	0	0	0
GAR	Broth	##	##	##	##	##	##	##	##	##	#	0
	Agar*	##	##	##	##	##	##	##	##	##	##	0

*Subculture from the corresponding broth tube after forty-eight hours. ##, Full growth; #, scant growth; 0, no growth.

The broth tubes were read at forty-eight hours, the agar plates at twenty-four hours.

As is evident a marked degree of drug resistance had been produced in GAR. Coincidentally with this, the phase of the organism had been changed, for GAR showed definite differences from the parent strain GA with regard to the colonies on blood agar plates. Comparing the two, GAR was smaller, had a finely stippled surface, and showed some roughening of the border, especially after forty-eight hours. GAR also showed some tendency to spontaneous clumping in saline suspensions as compared to none in GA. From this we inferred that GAR was in an intermediate stage between smooth and rough, an S-R form, while GA had remained smooth. This difference persisted and was even somewhat accentuated at the end of the experiments.

One month later GA and GAR were again tested, neither strain having been exposed to sulfanilamide in the interval. The findings were essentially the same as in Table I, the difference in susceptibility of the two strains to the drug being slightly more marked than before. An attempt was now made to modify the phase of GAR by growing it in enriched media. Twenty per cent ascitic fluid broth was used. The result was another variant, called GARA, which resembled the smooth type of organism in its colonial form except that unhemolyzed red blood cells were usually present immediately beneath the colony. Six weeks, and again three months, after the second test of GA and GAR all three strains were tested at the same time. The results of these and the original tests were represented in Chart 1.

At the close of these experiments GA was morphologically in the S or M-S phase, GARA was similar except for the unhemolyzed red blood cells beneath

the colony as noted before, and GAR was a definite S-R form very distinct in its colony type from either of the others. It is evident, therefore, that over a period of three months there was, in the case of GAR, a marked loss of resistance to sulfanilamide without any reversion to the standard or smooth type of this organism. It is also evident that in the case of GARA change of phase did not alter the susceptibility of the organism to sulfanilamide or cause any quicker loss of resistance to the drug than was the case with the parent strain GAR.

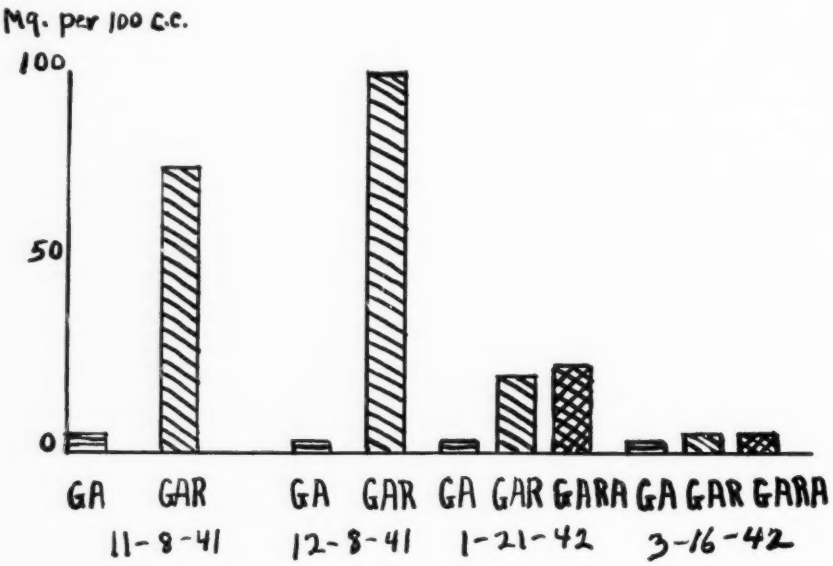

Chart 1.

SUMMARY

1. A high degree of resistance to sulfanilamide was produced in vitro in a group A hemolytic streptococcus.

2. This resistance was maintained at a high level for one month and subsequently spontaneously disappeared in the course of about three more months.

3. Evidence is presented which suggests that though change of phase and drug resistance may be produced simultaneously by the action of sulfanilamide these two factors are not dependent upon each other, and may be separately modified.

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CLINICAL EXPERIENCES WITH A MODIFICATION OF THE TAKATA REACTION IN BLOOD AND CEREBROSPINAL FLUID

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IN 1935 and 1936 I described a modification of the Takata serum reaction. This reaction had been advocated as a test for the diagnosis of liver diseases by Staub (1929) and Jezler (1929). The modified test, which I studied in 160 cases, has since been investigated by a number of workers in different countries. They have tested the sera of more than 2,500 patients suffering from different morbid conditions, including a large number of pathologic conditions of the liver. In the Italian literature the test is generally referred to as "Ucko reaction." It has also been applied to the cerebrospinal fluid of patients suffering from organic diseases of the nervous system and has given interesting results in comparison with other protein reactions. It seems, therefore, worth while to survey the experiences of these authors, some of which are published in journals not available in this country and which give sufficient data to judge the diagnostic value of the test. This is the purpose of this paper.

TECHNIQUE AND NATURE OF THE MODIFIED TEST

The reaction consists in a precipitation of serum protein by addition of sodium carbonate and mercuric chloride to serum.

Owing to an omission in the first publication (1935), a misunderstanding has arisen about the number of tubes required for the test. This is how it should be carried out:

Reagents: 0.36 per cent solution of *anhydrous* sodium carbonate; 0.5 per cent solution of mercuric chloride (sublimate). Into 5 clean test tubes of 11 mm. diameter each, containing 0.2 c.c. of serum, are measured 0.1, 0.15, 0.2, 0.25, 0.3 c.c. of the sodium carbonate solution. After shaking, the same quantities of the mercuric chloride solution are added and the tubes are shaken again. Readings by daylight are taken at once and after ninety minutes:

Negative reaction: three or more tubes are *translucent.* The mixture may be clear or may show a slight opacity.

Reaction +: the first three tubes are turbid and *not translucent.*

Reaction + +: all tubes show a uniform turbidity and are *not translucent.*

Reaction + + +: a thick *precipitation* in all the tubes forms *immediately* after the addition of the reagents.

The performance of the test is extremely simple. Only the two solutions have to be prepared with absolute accuracy. If kept in the dark and at 0° C., they remain accurate for a month, after which time they have to be renewed. They should be checked from time to time by adding a drop of phenolphthalein to a mixture of equal parts of the solutions. If accurate, the mixture shows a weak pink color. It is important always to use *anhydrous* sodium carbonate.

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The nature of the reaction has not yet been cleared up satisfactorily. The active part of the reagent seems to be an unstable complex oxychloride of mercury, which precipitates serum protein under special quantitative, and perhaps qualitative, conditions (Ucko, 1936-1938).

The precipitate formed is serum globulin while albumin has a protective influence (Ucko, 1936). The protein concentration and the albumin-globulin ratio are, therefore, of definite importance. Other still unknown factors may play a part.

THE TAKATA REACTION

Takata's reaction, which is read after twenty-four hours, consists of a flocculation produced by addition of a strong solution of sodium carbonate, mercuric chloride, and fuchsin to a progression of serum dilutions and was designed for quite different purposes. It was Jezler who showed that this reaction is strongly positive in the sera of cirrhosis of the liver. Jezler, however, used Takata's procedure without adapting it to the new purpose. Extensive clinical studies have proved that positive Takata reactions are most frequently found in the sera of patients with liver diseases, especially with cirrhosis of the liver. Positive reactions may, however, occur in other illnesses. Multiple myelomas (Kahler's disease) always give a strongly positive reaction. Positive reactions can also be found in cases of glomerulonephritis, nephrosis, pneumonia, pulmonary tuberculosis, etc.

My procedure has the technical advantage of giving a clear end point only after ninety minutes and of allowing a gradation of the results from + to +++. A comparison of the two reactions in 132 cases by myself (1936) and of 33 cases by Guglielmini (1937) showed that the modified reaction possesses a greater certainty in indicating affections of the liver.

RESULTS OBTAINED WITH THE MODIFIED REACTION

Results are summarized in Tables I and II. The total number of cases is approximately 2,600, 373 of these being patients with liver or gall bladder lesions.

All but one of 92 cases of *Laënnec's atrophic cirrhosis* (Table 1, *a*) had positive reactions (99 per cent). The reaction was +++ in 83 patients (90.2 per cent), ++ in 7, and + in one patient. Two of Cozzutti's cases had ++ reactions at an early stage. Statistics of the original Takata reaction (Schindel and Barth, 1934) have furnished only 83 per cent of positive and 6.8 per cent of doubtful (Jezler, 1938) results in liver cirrhosis.

Four cases of group *b* (Table I) had negative reactions: one with the diagnosis of chronic hepatitis (out of 4), one termed hypertrophic cirrhosis with splenomegaly (Takata's reaction negative), one hepatomegaly (Vaquez), and one hydatid cyst of the liver.

Fifteen of the 46 patients had +++ reactions. These consist of one patient with hypertrophic cirrhosis, one with amyloid degeneration of the liver, one with acute liver necrosis, 4 (of 16) with syphilitic hepatitis, one with arsenical hepatitis, one with liver abscess, one with multiple cysts of the liver, and 5 (of 7) with kala-azar. Regarding kala-azar, the formol test (gelatinization of the serum on addition of formol) may be remembered. It is due to a marked relative increase of the serum globulins (reduction of albumin?), a characteristic feature of kala-azar. Kala-azar has been included in this

group because the disturbed equilibrium of the serum proteins is probably due to the severe liver damage as a result of the protozoal infection.

One *reticulo-endothelioma* and 2 cases of *primary cancer* of the liver gave +, ++, and +++ reactions, respectively (Table I, *c*). The patient with a +++ reaction showed diffuse cirrhotic liver changes at autopsy.

TABLE I

	NO.	DIAGNOSIS	−	+	++	+++	AUTHORS
a.	92	Cirrhosis of the liver (Laënnec)	1*	1*	7	83	Boccia, Cozzutti, Di Benedetto, Guglielmini, Jezler,* Sacchetti, Ucko
b.	1	Hypertrophic cirrhosis	–	–	–	1	Boccia and Gamalero
	1	Hepatomegaly (Vaquez)	1	–	–	–	Sacchetti
	1	Hypertrophic cirrhosis + splenomegaly	1	–	–	–	Guglielmini
	1	Amyloid degeneration	–	–	–	1	Ucko
	1	Acute liver necrosis	–	–	–	1	Boccia and Gamalero
	1	Liver abscess	–	–	–	1	Boccia and Gamalero
	1	Hydatid cyst of liver	1	–	–	–	Boccia and Gamalero
	1	Mutiple cysts of liver	–	–	–	1	Boccia and Gamalero
	16	Syphilitic hepatitis	–	6	6	4	Benedetto, Cozzutti, Sacchetti, Ucko
	5	Enterogenous hepatitis	–	5		–	Benedetto
	4	Chronic hepatitis	1	2	1	–	Cozzutti
	1	Alcoholic hepatitis	–	1	–	–	Boccia and Gamalero
	1	Arsenical hepatitis	–	–	–	1	Boccia and Gamalero
	2	Amoebic hepatitis	–	–	2	–	Benedetto
	2	Hepatitis brucellaris	–	–	2	–	Benedetto
	7	Kala-azar	–	–	2	5	Benedetto
c.	2	Primary cancer of liver	–	–	1	1	Cozzutti, Ucko
	1	Reticulo-endothelioma	–	1	–	–	Cozzutti
d.	31	Secondary cancer of liver	9	8	10	4(2)*	Benedetto, Boccia, Cozzutti, Guglielmini, Jezler,* Sacchetti, Ucko
	1	Cancer of pancreas + jaundice	–	–	1	–	Cozzutti
	1	Cancer of bile ducts	–	1	–	–	Boccia and Gamalero
e.	55	Liver congestion (heart failure)	7	29	18	1*	Benedetto, Cozzutti, Jezler,* Sacchetti, Ucko
	1	Cardiohypertrophic cirrhosis	–	–	1	–	Boccia and Gamalero
f.	7	"Liver disorder?"	4	1	2	–	Ucko
	35	Cholecystitis	23	12	–	–	Sacchetti
	28	Gall bladder disease	24	4	–	–	Cozzutti, Ucko
	1	Obstructive jaundice (stone)	–	1	–	–	Cozzutti
	26	Gallstones + cholecystitis	–	12	12	2	Sacchetti
	6	Cholangitis	3	2	1*	–	Boccia, Sacchetti, Jezler*
g.	40	Simple jaundice	34	5	1	–	Boccia, Cozzutti, Guglielmini, Ornstein, Sacchetti, Ucko

*These results deviate from findings of majority of authors. See text.

Among 33 cases of *secondary carcinoma* of the liver of different origin (Table I, *d*), there were 9 negative and 4 +++ reactions. Twenty patients had + and ++ reactions. This gives a total of 24 positive reactions (73 per cent), while in a similar group of 85 cases only 30 per cent of positive Takata reactions were observed (Schindel and Barth, 1934).

All authors agree that the degree of positivity of the reaction in patients with *liver congestion* due to heart failure corresponds with the clinical condition (Table I, *e*). Only 7 of 56 cases gave negative reactions (12.5 per cent). In comparison, 44 of 45 patients with a failing heart without clinical signs of liver congestion had negative reactions (Cozzutti, Guglielmini).

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The next group (Table I, *f*) contains 103 patients, mainly with lesions of *gall bladder* and *bile ducts*. Fifty-four negative reactions were found. In 3 cases with gall bladder trouble and + reactions (Cozzutti) the liver was tender and enlarged and jaundice was present. Among the 4 cases labeled "liver disorder?" only the 3 patients with positive reactions showed signs of a lesion of the liver on examination.

Sacchetti's cases with 12 ++ and 2 +++ reactions will be discussed later.

The results obtained in cases of *simple* (catarrhal) *jaundice* (Table I, *g*) are important since most patients (34 of 40) had negative reactions. Very divergent results have been obtained with Takata's reaction in cases of jaundice. Six of my own cases had positive Takata and negative modified reactions. In some cases of jaundice positive results were the first signs of more severe liver damage (Ucko, 1936). No clinical details are available about the 6 positive cases in this group.

Di Benedetto, without quoting exact figures, states that most of his patients with simple jaundice had negative reactions, some, however, + and ++ reactions. His observations have not been included in Table I.

TABLE II

NUMBER		CLASSIFICATION	−	+ ++ +++
1.	230	Liver diseases and diseases with definite liver damage (Table I, *a-e*)	21 (9.1%)	49 51 104 (5) =90.9% =45%
2.	143	Diseases of the biliary system, uncertain liver cases, and simple jaundice (Table I, *f*, *g*)	88 (61.5%)	37 16 2 =38.5% =1.4%
3.	2,031 (approx.)	Other diseases	1,752 (Approx. 86.3%)	128 111 40 =13.7% =2%

Taken all together, 90.9 per cent of the cases with liver affections had positive reactions while only 38.5 per cent of positive results were obtained in patients suffering from diseases related to the biliary system and from simple jaundice. The number of +++ reactions in the first group was 104 compared with only 2 in the second group (45 per cent against 1.4 per cent). Also the percentage of ++ reactions is higher in group 1 than in group 2 (23 per cent against 11 per cent) (see Table II).

Patients suffering from all common internal and neurological illnesses, as well as a number of healthy people, have been examined by the different authors in order to find out how far positive reactions are related to affections of the liver. The total number of cases (2,031) is only approximate, since many authors do not quote the exact number of their patients.

Table III gives a list of nonhepatic diseases in which positive results have been obtained. They do not occur regularly. Many patients suffering from the same illnesses have negative reactions. The number of diseases is limited, and they are on the whole those in which the original Takata reaction has also been found to give positive results.

Of the patients examined 86.3 per cent had negative reactions, 11.7 per cent + and ++ reactions, and only 2 per cent +++ reactions. The majority of

patients with ++ and +++ reactions consisted of 104 cases of malaria observed by Vesce and of 25 skin diseases observed by Bronzini. The remaining cases are 17 with ++ and only 5 with +++ reactions; 3 patients with Kahler's disease (multiple myelomas), one with diabetes and one with miliary tuberculosis, all observed by Jezler and his collaborator, Bots. None of the other authors have found +++ reactions among their nonhepatic cases. The majority of these patients had + reactions (6.3 per cent).

TABLE III

NONHEPATIC DISEASES WITH POSITIVE REACTIONS

DEGREE OF REACTION +	++	+++
Nephritis, nephrosis	Nephritis, polyarthritis	Multiple myeloma (Jezler)
Diabetes	Rheumatoid arthritis	
Pernicious anemia	Diabetes	Diabetes (Jezler, Bots)
Peliosis rheumatica	Pernicious anemia	
Carcinosis, carcinoma of pancreas	Lymphogranuloma inguinalis	
Malignant lymphadenoma	Tuberculosis of lungs, miliary tuberculosis	Tuberculosis of lung (Rubino)
Tuberculosis of lungs, pleurisy	Lung abscess, bronchiectasis	Miliary tuberculosis (Jezler)
Pneumonia, lung abscess		
Typhoid, amoebiasis, brucellosis		
Malaria	Malaria, spinal tumor, tabes, general paralysis of the insane	Malaria (Vesce)
Cerebrospinal syphilis, hemiplegia	Tuberculosis of skin, other skin diseases	Skin diseases (Bronzini)
General paralysis of the insane (G.P.I.)		

DISCUSSION

The experiences of four authors could not be included in this general survey. An article by Montalbano (1938) on the subject was unobtainable. According to Vesce (1939), this author has examined 167 patients and has found positive results in most of his liver cases. He has, however, observed +++ reactions in patients without clinical signs of liver disease.

Backert's results (1938) have been omitted because he found only one negative reaction among 125 patients with different illnesses and 10 healthy persons. This fact can only be explained by an error in his technique. Backert himself is satisfied with his results, especially with regard to liver disease.

Ornstein (1937) has not recorded the degree of positivity of the reaction. Apart from the diseases in Table III, he has encountered positive results in cases with the diagnosis of pellagra, Grave's disease, and ovarian deficiency.

Rubino's (1938) readings of the reaction differ from those put forward by those mentioned and in previous papers. His results have, therefore, not been included in Table II and are dealt with separately.

Most authors come to the conclusion that the test is a very valuable, simple, and rapid laboratory method, generally indicating hepatic involvement and thus aiding clinical diagnosis.

Cozzutti emphasized that +++ reactions may occur in cases of syphilitic hepatitis and are not specific for liver cirrhosis. Table I shows other liver affections with +++ reactions.

Guglielmini regards the new reaction as being superior to the original one and believes that reaction +++ indicates cirrhotic changes; reaction ++, local-

ized liver affections; and reaction +, simple liver congestion. This classification is not confirmed by the result of the other authors.

Sacchetti followed up cases with chronic affections of the biliary system. His number of strongly positive reactions (++ and +++) is very high. According to him, the test is of great value in indicating possible damage of the liver parenchyma. Except in an emergency, patients with gallstones should not be operated on if their serum reaction is stronger than +. In such cases medical treatment should be given first. This may lead to an improvement of the test and to better chances if surgical interference is necessary. Two of Sacchetti's cases with +++ reactions died soon after operation. The postoperative recovery of cases with ++ reactions was unsatisfactory. After successful operations the reaction became negative.

Jezler, who introduced the Takata reaction into the clinic as a liver function test, and his assistant, Bots, are not in favor of modifications of the original technique. Bots' and Jezler's liver cases are identical and have, therefore, not been included separately in Table I. Jezler's results have been marked with an asterisk to show that they generally deviate from the findings of the majority of authors (– and + reactions in liver cirrhosis, +++ reaction in liver congestion, and ++ reaction in cholangitis). He also found +++ reactions in one patient with diabetes and one with miliary tuberculosis. With regard to the negative reaction in one patient with liver cirrhosis among 92 (Table I), it is interesting to note that a series of 25 Takata-negative cases, verified at autopsy, includes 2 cases of liver cirrhosis (Jezler, 1938, p. 431).

Although positive results with the test described are not confined to liver diseases, the great number of cases collected by at least 14 different authors show clearly the enormous incidence of positive reactions when the liver is primarily or secondarily involved in the morbid condition. Positive results in nonhepatic cases are comparatively rare. There is, however, no doubt that the reaction, like the original Takata reaction, can be positive in cases without clinical or pathologic manifestations of liver disease. The test is, therefore, not a specific indication of liver disease. It can only be used as such in connection with the clinical findings. It indicates an alteration of the serum proteins of a nature which is not properly understood, but it is possible to conclude that the liver must play an important part in the etiology of this alteration. If used with the necessary precaution and in connection with the clinical observation, and if certain other causes and diseases (Table III) have been excluded, the test is undoubtedly an easy method of great value for diagnosis, prognosis, and treatment of liver disorders, and for the differential diagnosis of doubtful cases. On the strength of the observations collected in this paper it seems justified to recommend the reaction for the routine examination of possible liver affections and as a test for the liver function with regard to protein metabolism. Reactions +++ suggest the presence of a severe parenchymatous lesion or destruction of the liver. In the majority of cases this lesion is cirrhotic in nature. Other severe liver lesions of an extensive but not always diffuse character can also give +++ reactions. Marked impairment of the protein-regulating function of the liver, recognizable by a positive serum reaction, may, however, occur without gross morphologic lesion. Even a strongly positive reaction is reversible and can be affected by treatment, as in the case

of syphilitic hepatitis and liver congestion. The test is also of assistance for the differential diagnosis of jaundice since the majority of cases of simple jaundice give negative reactions. Cases of jaundice with ++ or +++ reactions should be diagnosed with caution. All my own cases with such reactions turned out not to be simple jaundice. The differential diagnosis between simple jaundice and mild hepatic necrosis, often impossible for the clinician, seems also to be facilitated by the test (see also Ucko, 1938).

TABLE IV

NUMBER	DIAGNOSIS	RESULTS	EFFECT OF TREATMENT
21	Acute and generalized exudative eczema	+++ 10 cases	Cured
10	Eczema rubrum madidans	++ 9 cases	Improved
5	Papulous eczema	+ 2 cases	Not improved
39	Different chronic eczemas	+++ 3 cases	Improved
10	Hyperkeratotic eczema of palms and soles	++ 3 cases	Cured
15	Squamous eczema	+ 5 cases	Not improved
100		32	

The occurrence of positive reactions in nonhepatic diseases suggests that alterations of the serum proteins, similar to those found most regularly in liver diseases, can take place in certain other illnesses (Table III). Some of these (e.g., diabetes, pernicious anemia, typhoid, malaria, amoebiasis, brucellosis, syphilis) even affect the liver morphologically. The involvement of this organ may account for the disturbance of the protein metabolism manifesting itself in a positive serum test. Other diseases (e.g., nephritis; Kahler's disease; focal, acute, and chronic infections like rheumatoid and other forms of arthritis; pneumonia; pleurisy; lung abscess; bronchiectasis; tuberculosis; lymphogranuloma) show no such implication of the liver. A disturbed protein metabolism in Kahler's disease, in renal and certain infectious diseases is, however, known. It has not been recognized until recently that the liver plays an important role in the regulation of protein metabolism. Liver function tests (glucose, levulose, and galactose tolerance, hippuric acid formation, etc.) have shown impaired liver function in illnesses where no hepatic involvement was expected. There is strong evidence that the liver is responsible for the alteration of the serum proteins in hepatic diseases. While the matter is open to discussion, it is very likely that the liver is also responsible for the disturbance of the protein metabolism in those nonhepatic diseases where such a disturbance is a regular occurrence. The alteration of the serum proteins, which leads to a positive serum reaction, would then be the manifestation of a liver disturbance brought about by the causative agent. Apart from its practical significance the test, therefore, draws attention to the liver in diseases with disturbed protein metabolism and to the role of this organ in protein synthesis and equilibrium.*

SPECIAL INVESTIGATIONS

Some authors have used the test for determining the liver function in certain illnesses. Their results merit special consideration.

*Recent investigations with the cephalin-cholesterol flocculation test (Hanger, F. W.: J. Clin. Investigations **18**: 261, 1939; Rosenberg, D. H.: Arch. Surg. **43**: 231, 1941) give results almost identical with those obtained by the modified Takata test, and it will be interesting to compare both reactions on the same cases. The similarity of the results of the two different tests in certain nonhepatic diseases points to a liver dysfunction rather than to an unspecific alteration in such cases.

1. *Malaria.* Vesce (1939) examined 137 cases of African malaria. The incidence of positive reactions was surprisingly high. He found 82 ++ and 22 +++ reactions. Provocation by injection of a concentrated liver extract and adrenalin and by violent physical exercise had no appreciable influence on the results. To explain the great number of positive results, Vesce puts forward the theory that a positive reaction indicates a functional disturbance of the reticulo-endothelial cells rather than of the liver cells, the former being permanently damaged by the malarial infection. This theory is interesting and could perhaps be further substantiated by experimental damage to the reticulo-endothelium without involving the liver cell, but the strongly positive reaction in cirrhosis of the liver with extensive destruction of the liver parenchyma and, on the other hand, a weakly positive reaction (+) found in a case of reticulo-endothelioma (Table I, *c*) does not render this conception very probable.

2. *General paralysis of the insane.* Angrisani (1937) examined the sera of 95 cases of general paralysis of the insane. Fifty-four patients had negative reactions, 3 had +, 2 had ++ reactions. No +++ reaction was found. The five patients with positive reactions all had clinical signs of liver damage, either of specific or postmalarial origin. Similar results were obtained by the author with van den Bergh's and with Bengal red test. He concludes that liver damage in general paralysis of the insane, occurs rarely and disputes the observations of Cassiano (1935), who found frequently positive results using the original Takata reaction.

3. *Pregnancy.* The majority of pregnant women examined by Zambonini (1938) had negative reactions. Among 120 cases of normal pregnancy only 11 (9 per cent) had a + reaction. No ++ or +++ reactions were found. Reactions + occurred most frequently during the third and sixth months of pregnancy. There was no difference between primiparas and multiparas. Unfortunately, no examinations were made in cases of pathologic pregnancy or toxemia.

4. *Skin diseases* (Eczema). Bronzini (1938) studied the reaction in 100 cases of different eczematous conditions. The number of strongly positive reactions is unusually high and throws doubts on his experimental technique. Patients with positive tests were also treated for possible liver damage, but the author does not state anything about the nature of this treatment. His results are included in Table II and are in detail in Table IV. If they can be confirmed by others, they would represent an important contribution to our conception of eczematous conditions.

5. *Lung tuberculosis.* Rubino (1938), who examined 200 cases of pulmonary tuberculosis, compared the results of the test in 36 patients with the amino acid curve in the blood after ingestion of glycine, a procedure which is used as a liver function test. The author does not give exact figures. He states that 30 per cent of his cases had negative reactions, 30 per cent had + and ++ reactions, and 40 per cent had +++ reactions. The latter include "immediate and delayed +++ reactions," but since only an immediate precipitation can be regarded as a +++ reaction, Rubino's classification of results may be ignored. Positive reactions in cases of lung tuberculosis have been found by other authors but never in such a high percentage of cases. Rubino states that the degree of positivity in his series corresponded with the severity of the tuberculous lesion. He, therefore, regards the test as a useful addition to other

methods for diagnosing the degree of activity of the tuberculous process. He noted that the result of this test and tests of the blood sedimentation rate were usually parallel. This fact has not been observed by other authors. He found, however, positive reactions with a normal sedimentation rate. The glycine reaction showed disturbed liver function in an unspecified number of cases, but the author's reaction corresponded with the glycine test in only 50 per cent of the cases. Rubino concludes that the reaction furnishes no indication of a liver deficiency: he disregards the fact that both tests measure different types of liver function. The importance of his observations lies in the close relationship he found between positive reactions and the severity of the tuberculous lesion and in the demonstration of a damaged liver function in cases of lung tuberculosis. Disturbances of protein metabolism in cases of severe tuberculosis have been demonstrated earlier; the albumin fraction of the serum is generally diminished; the globulin fraction, and especially fibrinogen, is increased. This may well be due to damage to the liver and impairment of its regulation of protein metabolism.

THE CEREBROSPINAL FLUID

Takata and Ara (1936) applied the original test to the cerebrospinal fluid and found it to be positive mainly in cases of syphilis of the nervous system. The Takata reaction has, however, not become a routine test for the examination of the cerebrospinal fluid because it had no advantages in comparison with other protein reactions and the results were not found to be specific for syphilis.

Three authors studied the new modification of the test on the cerebrospinal fluid. Zanetti's paper (1938) "Neurosyphilis and Ucko Reaction" was not obtainable in this country.

Ornstein (1937) examined the cerebrospinal fluid of 46 cases of general paralysis of the insane, and of 12 cases of tabes and compared the results with those of the Wassermann and Meinicke tests. The cerebrospinal fluid of 22 patients with negative Wassermann and Meinicke reactions were tested in comparison. His results are summarized as follows:

NUMBER	DIAGNOSIS	WASSERMANN		MEINICKE		UCKO	
		+	−	+	−	+	−
46	General paralysis of the insane	46	−	46	−	46	−
12	Tabes	8	4	8	4	8	4
22	Other affections	−	22	−	22	−	22

These data show a complete correspondence of the three reactions, and Ornstein concludes that the test is more valuable than Meinicke's and other reactions owing to its simple technique.

A very important contribution was made by Fleischhacker (1938). This author used only one tube, adding the lowest concentration, i.e., 0.1 c.c. of each reagent to 1.0 c.c. of cerebrospinal fluid. If the cerebrospinal fluid is free from blood and is not contaminated by bacteria, a positive reaction (marked opacity or precipitation) appears between forty-five and ninety minutes. Fleischhacker examined 153 cases of treated and nontreated general paralysis of the

insane and 24 different nervous and mental disorders and compared the results with the reactions of Weichbrodt, Pandy, Lange, and Wassermann.

In 61 cases of general paralysis of the insane all the reactions were positive. In 92 treated cases the results were as follows:

UCKO		LANGE AND PANDY		WASSERMANN		SERUM—WASSERMANN	
+	−	+	−	+	−	+	−
36	36	0	92	10	82	31	61

Twenty-four nonsyphilitic cases gave the following reactions:

DIAGNOSIS	UCKO		LANGE		PANDY	
	+	−	+	−	+	−
Cerebral tumor	2	−	−	2	−	2
Cerebral thrombosis	1	−	−	1	−	1
Disseminated sclerosis	1	−	−	1	−	1
Mental deficiency	3	3	−	6	−	6
Organic disorder?	7	4	−	11	2	9
Angioma cerebri	−	1	−	1	−	1
Posttraumatic psychosis	−	1	−	1	−	1
Fracture of skull	−	1	−	1	−	1

It can be concluded from the results that the reaction is not specific for syphilis but indicates generally abnormal protein conditions in the cerebrospinal fluid. Without being hypersensitive the test is more sensitive and, therefore, superior to Pandy's protein reaction usually made in the routine examination of the cerebrospinal fluid. The reaction may still be obtained—as in the case of treated general paralysis of the insane—when all other findings are apparently negative. It would be of interest to test cases of mental disorders, since Fleischhacker found positive reactions in cases of mental deficiency indicating lesions not detectable with the usual methods.

SUMMARY

The experience of different authors on approximately 2,500 cases with a simple and rapid modification of the Takata reaction is summarized and discussed.

Ninety per cent of patients suffering from liver diseases had positive reactions while at least 86 per cent of negative reactions were encountered in patients suffering from other illnesses. The test which indicates an alteration of the serum proteins seems to measure the liver function with regard to protein metabolism. Certain nonhepatic diseases frequently, but not always, give positive reactions. The problem of whether a positive test in these illnesses means a disturbance of the protein-regulating function of the liver or whether it occurs independently is discussed.

The experience of a number of authors with the test in cases of general paralysis of the insane, pregnancy, skin diseases, and tuberculosis is given in detail.

The reaction, which has also been applied to the cerebrospinal fluid, seems to be more sensitive than the routine tests for protein. It will, therefore, be of value in detecting minor alterations of the cerebrospinal fluid in disorders of the nervous system.

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THE EFFECT OF ASCORBIC ACID (VITAMIN C) ON THE SENSITIVITY TO SALICYLATES IN A CASE OF RHEUMATIC FEVER

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A SEVERE case of rheumatic fever that rapidly became intolerant to salicylates was observed recently. The intolerance was manifested by a marked ringing in the ears that continued in spite of the reduction of the drug to a very low and ineffective amount. At about the same time, the occurrence of several severe nosebleeds, as well as a positive tourniquet test, and a low plasma ascorbic acid content, suggested the use of large amounts of ascorbic acid. The previous diet was analyzed and was found to be markedly deficient in vitamin C. Dramatically enough, the nosebleeds and the severe tinnitus ceased after forty-eight hours of vitamin C therapy and did not return, although the salicylates were increased to the initial high level and were continued in this manner for ten additional days.

Some observers[1] have pointed to a deficiency of vitamin C in acute rheumatic fever. This deficiency may predispose the person to toxic reactions to certain drugs, e.g., in this case salicylates. In any event, the toxic reaction ceased when vitamin C was given in adequate amounts.

CASE REPORT

P. V., a 15-year-old male, suddenly developed a high fever (104° F.); rapid pulse rate (130), pain in both shoulders, and dyspnea. The boy had no history of any previous serious disease and was in excellent health until the onset of the present illness. The mother is a rheumatic cardiac with both mitral and aortic murmurs, and a recent history of an attack of myocardial failure.

Examination of the patient revealed a rough, to-and-fro leather-like murmur over the base and apex of the cardiac area. There were no signs in the lungs or elsewhere. There was no swelling, redness, or limitation of motion in the shoulder joints, or in any other joints of the body. The pain in the shoulder joints was apparently due to a reflex mechanism. The to-and-fro pericardial friction rub was evanescent, lasting only a few hours. The sedimentation time was extremely rapid, twenty minutes for an 18 mm. fall. The blood count was white blood cells 15,200, polymorphonuclear leucocytes 85 per cent, lymphocytes 12 per cent, monocytes 3 per cent, hemoglobin 85 per cent, red blood cells 4,350,000. A diagnosis was made of rheumatic carditis.

Sodium salicylate was started at the rate of 15 grains (1 Gm.) every four hours combined with 5 grains (0.3 Gm.) of sodium bicarbonate. In addition, 60 grains (4 Gm.) of sodium salicylate was given by rectum once a day. On the third day of illness and salicylate medication, the patient developed a nosebleed and severe ringing of the ears. During the next two days the tinnitus continued severely in spite of the reduction of salicylate medication to 15 grains a day by mouth. The nose bled profusely during this time. A tourniquet test showed twelve petechiae in a 2.5 cm. circle after fifteen minutes of positive pressure. The plasma ascorbic acid content was 0.4 mg. per 100 c.c. (Farmer and Abt[2] method—normal value equals 0.7 mg. per 100 c.c.) It was, therefore, decided to give the patient large amounts of vitamin C. In addition, the diet of this patient over the preceding four weeks was reviewed and was found to be markedly lacking in foods containing vitamin C.

One hundred milligrams of ascorbic acid were given by mouth three times a day. Within forty-eight hours the nosebleed, as well as the ringing in the ears, had ceased. The salicylates were then stepped up to the former doses without development of tinnitus, or indeed

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any other sign of intolerance. The combined medication was continued for ten days without any ill effect. Urine examination was entirely negative for signs of renal irritation. The patient made an uneventful recovery, with a residual mitral systolic murmur as a sequel.

COMMENT

There is some evidence to show that in the guinea pig, chronic scurvy with a superimposed infection of β-streptococci produces lesions similar to those found in acute rheumatic fever.[1, 3] Scurvy alone does not produce the characteristic lesion. Clinically also, the ascorbic acid level of the blood plasma was found to be low in a group of patients[1] with rheumatic fever. It can easily be shown that rheumatic fever is a disease of the poor and undernourished,[4] and also that latent scurvy and rheumatic fever occur late in winter and early in spring, during the very same period that vitamin C is less widely available in foods.[5] However, the treatment of rheumatic fever patients with vitamin C in adequate amounts has proved disappointing.[6] Perla and Marmorston[7] believe that this does not rule out the connection between vitamin C and rheumatic fever, for vitamin C deficiency might enable the streptococcus to gain a foothold in these patients. Once this has been attained, vitamin C itself cannot affect the pathologic picture or prevent a recurrence. These authors feel that there exists some relationship between the lower vitamin C content of the blood and rheumatic fever, even though the connection has not been incontrovertibly proved as yet.

There also appears to be much evidence that an excess of vitamin C is a prophylactic against the toxic effects of neoarsphenamine and some other drugs. Vitamin C has been used with success in the control of reactions of certain patients to neoarsphenamine. In these persons, there may have been an insufficiency of the vitamin in the diet, and its value may be due to this fact. Recently, Marin[9] observed cases of mercurial and bismuth stomatitis among syphilitic patients, and administered ascorbic acid since it was decreased in the blood and urine. The ascorbic acid cured ten cases of this complication.

In this study the presence of a large amount of vitamin C in a patient who had a low serum ascorbic acid level, and other evidences of vitamin C deficiency appeared to remove the evidence of salicylate toxicity (marked tinnitus). At the same time marked nasal hemorrhages ceased wtihout recourse to packing or cauterization. It is suggested by one author that the hemorrhagic manifestations of rheumatic fever are due to a vitamin C deficiency.[1] In the study of bismuth and mercury sensitivity cited,[9] the author stated that vitamin C was necessary for the metabolism of these substances in the body; if it was not present in sufficient amounts toxicity would supervene. This may also be true in the case of salicylate sensitivity. This is suggested by the following simple experiment:

EXPERIMENT

To a test tube containing 5 drops of 1 per cent methylene blue solution in 5 c.c. of water a few crystals of ascorbic acid are added. On heating the tube the blue solution is immediately decolorized and a white solution results. The methylene blue is apparently reduced by the ascorbic acid. To another test tube containing 5 drops of 1 per cent methylene blue solution in 5 c.c. of

water, some sodium salicylate crystals are added, and the resulting mixture is allowed to stand. Even though vitamin C is later added in large amounts, as high as 50 to 100 mg., and the resulting solution is heated, no decolorization results. The vitamin C has apparently been fixed by the sodium salicylate and the methylene blue is not reduced.

It is possible that in the body sodium salicylate has an affinity for ascorbic acid and binds it, so that less and less is available for other functions. However, this conclusion cannot be drawn from the foregoing simple experiment.

Whatever the relationship of the lack of ascorbic acid to the toxicity of sodium salicylate, it would seem to be worth while to study the problem further, to see whether it is widely applicable. Certainly it can be said that in this case, saturation of this patient with large amounts of vitamin C (normal daily requirement is approximately 50 mg.)[10] enabled the continuation of salicylate medication and appeared to have a salutory affect on the nosebleed.

CONCLUSIONS

1. A severe case of rheumatic carditis became intolerant to sodium salicylate early in the course of the illness.

2. A low plasma ascorbic acid content, positive tourniquet test, and severe nosebleed suggested the presence of vitamin C deficiency.

3. The preceding diet of this patient proved to be deficient in vitamin C.

4. After the vitamin C intake was increased, sodium salicylate was again given in large amounts with impunity.

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EFFECT OF TRASENTIN A, TRASENTIN, AND MORPHINE ON RESPIRATION*

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IT HAS been shown that trasentin is antagonistic to the actions of morphine on the bladder,[1] and recent experimental evidence has furnished confirmation of such action in regard to trasentin A on the isolated ureter.[2]

As it is possible that trasentin and its chemical allies are capable of inhibiting certain morphine effects other than on the bladder and ureter, it seemed of interest to investigate the relationship of effects on respiration using trasentin, trasentin A, and morphine in various combinations. Morphine and trasentin are both used therapeutically in certain combinations, such as ureteral colic.

PROCEDURE

In this work, 58 experiments were performed on dogs and 21 on cats. Respiration was recorded by means of a blunt intrapleural trocar connected to a sensitive tambour. The details of this simple, but very satisfactory, device are shown in Diagram 1. This apparatus may be used on unanesthetized as well as on anesthetized animals. When used in the former instance, a small amount of 1 per cent procaine hydrochloride is injected beneath the skin between the fifth and sixth interspace in the midaxillary line. A very small incision is then made through the skin with a scalpel, and the trocar is pushed with a craniad motion gently, but firmly, through the intercostal muscles and parietal pleura into the pleural cavity. It is very easy to accomplish this procedure without puncturing the lung, since the trocar falls into the space between the parietal and visceral pleura when resistance of the parietal pleura is overcome. Immediately upon entering the pleural cavity, respiratory excursions are recorded, provided no air has entered the cavity during the introduction of the trocar. To insure the prevention of pneumothorax, suction is applied to the T-tube after insertion and before withdrawal of the trocar. If air entered during the insertion of the apparatus and is removed by suction, it takes about three to four minutes for equilibrium to become established. We feel that this method is an excellent one to record direct respiratory movements, since there are no

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Trasentin A is phenylcyclohexyl acetic acid ester and is available only for experimental use. It is now designated as trasentin 6-H.

Trasentin is diphenylacetic acid diethylaminoethanol ester.

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by-passes or hindrances and there is no lag in the recording of all respiratory waves. This method also has the advantage of being used many times for the same animal, thus making each animal his own control.

We at first tried without success to slow respiration with large doses of morphine given subcutaneously or intravenously in unanesthetized dogs. Finally, we made use of a standard technique of anesthetizing the dog with 26.1 mg. per kilogram of pentobarbital. As soon as the anesthesia was complete, the animal was placed on the table and the trocar was inserted and connected to the tambour. After suitable control respiratory records were made, 5 mg. per kilogram of morphine were injected intravenously. This method gave uniform slowing of respiration.

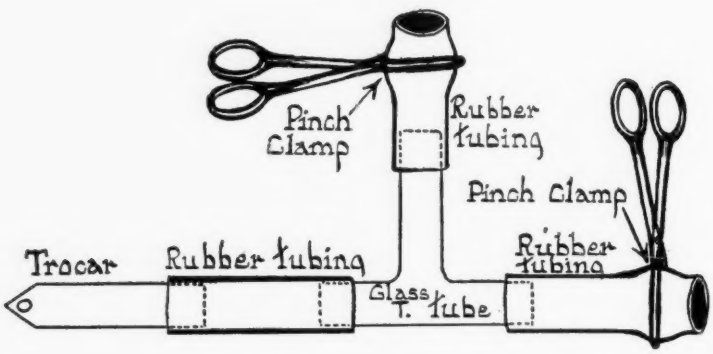


Diagram 1.—A schematic representation of the intrapleural trocar used in the experiments to record respiration.

In a certain number of acute experiments blood pressure and respiration were recorded simultaneously. Anesthesia was induced by pentobarbital, or barbital urethane administered intraperitoneally. All the cats and a few dogs used in this study were so treated.

As a rule, 6 mg. per kilogram of trasentin and 2 mg. per kilogram of trasentin A were given intravenously.

A few experiments were done on blood pressure alone, and the effects of trasentin A with and without intact vagi were compared.

RESULTS

In general, the results indicate that trasentin and trasentin A prevent depression of respiration by morphine when they are given before, after, or with morphine. When the trasentins are given with morphine, they never add to the depression of respiration produced by this opiate.

Fig. 1 illustrates well the effects of trasentin A and morphine on respiration of the dog. As noted on the tracings, these animals received pentobarbital previously.

Fig. 1*A* shows that 5 mg. per kilogram of morphine were given intravenously and (usually almost at once) marked depression of respiration ensued

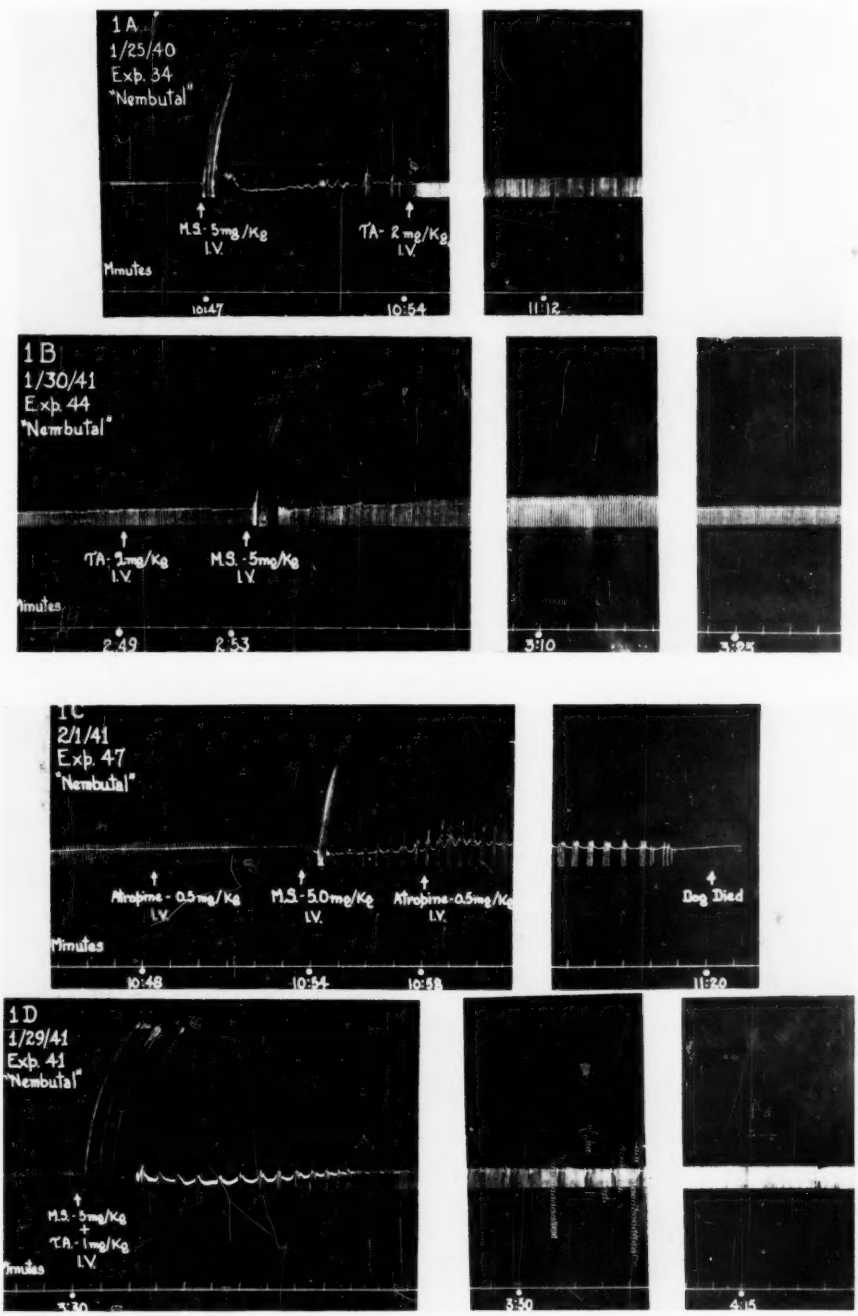


Fig. 1.—*A*, This tracing exhibits the effectiveness of trasentin A to counteract the depressant effect of morphine on respiration. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T.A. = trasentin A.

B, This tracing illustrates the ability of trasentin A to prevent the depressant effects on respiration by morphine. Both drugs were given intravenously.

C, This tracing illustrates the failure of atropine not only to prevent but also failure to correct depression of respiration by morphine. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate.

D, This tracing presents evidence that when morphine and trasentin A are given together the depressant effects of morphine on respiration are reduced. This was a smaller dose of trasentin A than that usually employed. Both drugs were given intravenously.

Note that in tracings shown in Fig. 1*A-D* there was at first a brief but sharp increase in respiration when morphine was administered.

following the brief stimulation. (This latter effect will be commented on in the discussion.) When 2 mg. per kilogram of trasentin A by intravenous injection were then given eight minutes later, the respiration returned to an amplitude and rate greater than the normal rhythm preceding the injection of morphine.

Fig. 1*B* shows that 2 mg. per kilogram of trasentin A administered intravenously four minutes before 5 mg. per kilogram of morphine entirely prevented depression of respiration by morphine. This is a very important finding since a large percentage of animals died after receiving such a dose of morphine alone. When one compares this preventative and corrective action of trasentin A with that of atropine, the difference is very striking. Fig. 1*C* demonstrates not only the failure of atropine in a dose of 0.5 mg. per kilogram intravenously to prevent the depressant effect of morphine on respiration, but also its failure to correct the depressant effect of this hypnotic as well. As a consequence, the animal died from the effects of the morphine. This was the usual finding in these experiments.

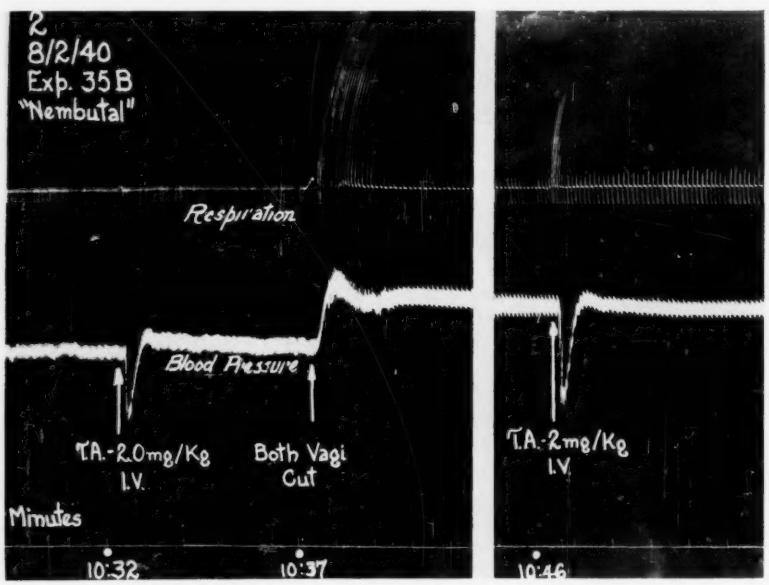
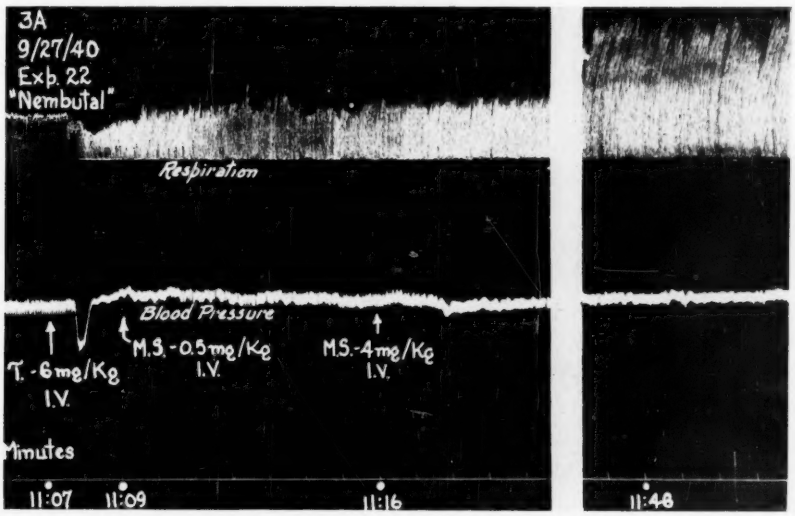


Fig. 2.—This tracing illustrates the effect of trasentin A on respiration and blood pressure. When given intravenously by itself, note the increased amplitude of respiration. The fall in blood pressure resembles that of acetylcholine and is present whether the vagi are present or divided. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T.A. = Trasentin A.

Fig. 1*D* shows a combination of 5 mg. per kilogram of morphine plus only 1 mg. per kilogram of trasentin A administered intravenously. Even with this small dose of the latter drug, the depressant effect of morphine on the respiration lasted only about nine minutes, and thereafter the respiration returned to above the normal rhythm when compared with the rate and amplitude before these drugs were given. This clearly demonstrates the protective action of trasentin A.

In other instances large subcutaneous doses of morphine alone were given and marked depression of respiration followed. Here, as already noted, trasentin A corrected the inhibitory effect of the hypnotic.



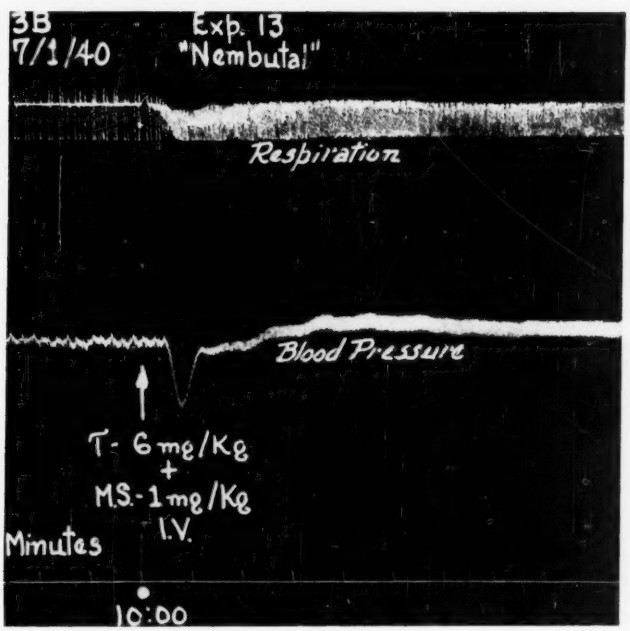


Fig. 3.—*A*, This tracing illustrates the effect of trasentin in preventing depression of respiration by morphine. Note the acetylcholine-like fall of blood pressure produced by trasentin. Both drugs were given intravenously. Time trace, minutes. Kymograph speed, 15 mm. per minute. M.S. = morphine sulfate. T. = trasentin.

B, This tracing presents the constant finding of stimulation of respiration when trasentin and morphine are administered intravenously as a single dose. Note that the blood pressure did not fall to any greater extent than when trasentin was given by itself, as seen in Fig. 3*A*.

Results similar to these were obtained in the cat, but the effectiveness of trasentin A was not as marked, since in our hands morphine alone tended to stimulate respiration. However, it has been shown that large, repeated doses of morphine will produce respiratory depression in the cat and evidence of the ability of trasentin A to prevent this depression has been obtained.

Simultaneous records of blood pressure and respiration were made under pentobarbital anesthesia (Fig. 2). In this tracing 2 mg. per kilogram of trasentin A given intravenously produced an acetylcholine-like fall in blood pressure. The vagi were then divided, and the trasentin A administration was repeated. The fall in blood pressure was still present and of the same magnitude, and it is seen that the amplitude of respiration is improved. Other experiments using doses of atropine which would paralyze the vagi before the administration of trasentin gave similar results. These results indicate that trasentin A acts beyond the vagus.

Effects with trasentin and morphine were of a slightly different nature than with trasentin A and morphine, although they pointed in the same direction. Fig. 3*A* shows the effect of 6 mg. per kilogram of intravenous trasentin on a dog that was anesthetized with pentobarbital. The blood pressure change is similar to that of trasentin A, but the fall is not as great. This is of interest, since the dose of trasentin used was three times that of trasentin A. As noted in this tracing, the respiration was stimulated and successive doses of 0.5 and 4 mg. per kilogram of morphine given intravenously failed to produce any depression. This result, however, was not as consistent as that noted with trasentin A. Fig. 3*B* illustrates the most significant and constant effect of trasentin. Here intravenous trasentin and morphine given together always produced stimulation of respiration. In this instance, only 1 mg. per kilogram of morphine plus 6 mg. per kilogram of trasentin was used, but the latter dose in combination with larger doses of morphine (up to 6 mg. per kilogram) gave a similar effect. It will be noted that the combination of morphine and trasentin, as seen in Fig. 3*B*, gives a fall in blood pressure of no greater depth than the trasentin alone, as noted in Fig. 3*A*.

DISCUSSION

These results clearly indicate that trasentin A and trasentin when administered before, after, or with morphine do not add to any depression of respiration caused by morphine. This correlates well with the previously described effects of the trasentin on the activity of the bladder[1] and the ureter.[2] Hence we feel that *experimental* evidence in animals does not indicate that it is dangerous to give these drugs to human beings with or following morphine.

Maloney and Tatum[3] have reported that morphine increases the effectiveness of inhibitory vagal impulses on respiration in the rabbit. This suggests that morphine might depress respiration at least partly by a central, cholinergic action on the vagus. The possibility that morphine possesses central cholinergic actions has been postulated.[4] This work concerned potentiation of morphine relief of pain by prostigmine.[4] Since this potentiation occurred and since morphine acts centrally, it was suggested that morphine had a central cholinergic effect. That there is a good possibility that central cholinergic transmission occurs has been suggested by Adam and others.[5] Since the trasentins are believed to be anticholinergic in activity as well as antimyonergic on smooth muscle, they may counteract morphine depression of respiration in this fashion. It is, of course, possible that they may also stimulate the respiratory center.

When one compares the effectiveness of the trasentins on respiration depressed by morphine, trasentin A (on basis of dosage) is three times as efficient.

Graham and Lazarus,[8] Meier and Hoffmann,[9] as well as some unpublished studies of this laboratory suggest that trasentin A has a more "atropine-like" action. This might suggest that the efficacy of trasentin A had to do with this type action, but certainly our results do not bear out any efficient action of atropine on morphine-depressed respiration.

We were interested in blood pressure effects of the trasentins, since their actions on respiration were stimulatory. In this regard both drugs gave an acetylcholine-like drop in blood pressure. Trasentin A gave about twice as great a fall as did trasentin and only one-third the dose of the latter was used in comparing it with the effects of the former drug. Apparently this action on the blood pressure is not central, since it occurs after vagotomy and after the vagus is paralyzed by atropine. This suggests that its effect may be on the smooth muscles of the arterioles or on the heart itself. The precipitous drop with rapid return to normal suggests the former point of action.

Our work confirms the reports of Maloney and Tatum,[3] and Barlow and Gledhill[6] concerning the synergistic effects of barbiturates and morphine. A note of warning is not out of place in regard to the use of barbiturates and morphine indiscriminately in the human being.

A constant unexplained phenomenon observed was the brief stimulation of morphine on respiration when this drug was given intravenously to dogs. This occurred with or without anesthetic doses of pentobarbital. It was usually accompanied by a "cry" and slight convulsive movements. While the research of Plant and Pierce[7] has shown that large subcutaneous doses of morphine in dogs will cause convulsions with increased respiration, we have never seen a report of such effects with such small intravenous doses as we used. This effect might be explained on the basis of a brief, massive bombardment of the brain by morphine sulfate causing this stimulation. Such an effect should always be considered in giving intravenous morphine to patients. It may be that the susceptibility seen in some human beings is a reflection of such an effect.

SUMMARY

1. Trasentin A or trasentin given after or before morphine, or combined with it, stimulate respiration depressed by morphine and prevent morphine from depressing respiration in dogs and cats.

2. The trasentins cause an acetylcholine-like fall of blood pressure in anesthetized dogs.

3. On the basis of this work the trasentins are as antagonistic to the respiratory depressant effect of morphine as they are to actions on the bladder and ureter.

4. Suggestions are made as regards the clinical use of morphine and trasentin, morphine and barbiturates, and intravenous administration of morphine.

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FURTHER STUDIES ON GLUCOPHYLLINE*

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IN A PREVIOUS report on the diuretic activity of glucophylline by Maloney, Burton, and Robinson,[1] in which experimental data were presented regarding the action of this double salt of theophylline and methylglucamine, a xanthine compound exhibiting the very desirable properties of high diuretic efficiency with long duration of action, it was indicated that our study was being continued with the object of determining "whether glucophylline exerts any untoward effects on the liver or kidney." The present report sets forth these and other pertinent results obtained from this investigation. The study was conducted in three parts, viz., the effect on (1) weight, (2) blood, and (3) vital organs. Full-grown dogs and immature rabbits were employed.

1. Weight. A. Dogs: Nine full-grown dogs, ranging in weight from 8.2 to 11.5 kg., were divided into groups of three and treated with physiologic saline (controls), methylglucamine, and glucophylline, respectively. All the animals lost weight during the course of the fifty-four days of the experimental period. The percentage loss in each group was 6.7, 9.3, and 11.6 in the order named. We do not think the medicaments played any significant role. The determinants here were probably the new diet and the unaccustomed artificial environment of the kennels (see Table I).

B. Rabbits: Thirty-six female rabbits, ranging in weight from 0.8 to 1.7 kg., were employed. These were divided into three groups of twelve. Half of the animals in group 1 received no treatment whatever, while the other half received normal physiologic saline. These were used as two sets of controls. Those in groups 2 and 3 received methylglucamine and glucophylline, respectively. For convenience, all animals received equal volumes of the substances injected: the rabbits received 0.2 c.c. for the first period of thirty

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days and 0.4 c.c. for the second period of thirty days; the dogs, 1 c.c. throughout the duration of the treatment. The methylglucamine was prepared as a 6.2 per cent solution, so that each rabbit received 12.4 mg. and each dog 62 mg. of this salt per dose; the glucophylline was prepared as a 10 per cent solution (in the formula the ratio of methylglucamine to theophylline is 1:16 to 1:18), so that each rabbit received approximately 10 mg. for thirty days and 20 mg. later, and each dog received approximately 50 mg. of each of the two constituents in the compound. On the basis of the initial weight the milligram per kilogram dose was then computed for each animal. This figure appears in the respective tables. Injections were all made intraperitoneally once daily. All the animals in the two species were kept under similar environmental conditions and were provided at all times with an excess of food and water. Weights were recorded at weekly intervals. With the exception of one control rabbit, which died on the twenty-fourth day of the experimental period, all animals lived to the end and without exception appeared to maintain normal health throughout. The dogs in the glucophylline group (No. 3) were definitely more

TABLE I

WEIGHT CHANGES IN DOGS

GROUP	ANIMAL	DOSE TOTAL	INITIAL WEIGHT (KG.)	54 DAYS FINAL WEIGHT (KG.)	% DECREASE	WEIGHT LOSS (KG.)
Control (Saline)	1	1 c.c.	8.3	7.7	7	0.6
	2	1 c.c.	11.4	10.9	4	0.5
	3	1 c.c.	11.1	10.1	9	1.0
Methylglucamine hydrochloride	4	6 mg./kg.	10.3	10.2	1	0.1
	5	6.7 mg./kg.	9.2	8.0	13	1.2
	6	5.4 mg./kg.	11.5	9.9	14	1.6
Glucophylline	7	10.8 mg./kg.	9.2	6.93	25	2.3
	8	10.1 mg./kg.	9.9	9.6	3	0.3
	9	10.3 mg./kg.	9.8	9.1	7	0.7

active and lively than the others. It is of interest to note that the animal caretaker, who had no knowledge of what the animals being injected were receiving, volunteered the observation that the group of rabbits which we were treating with glucophylline ate more food and drank more water than those making up the other group; and that the amount of feces and urine voided by these animals was considerably greater. Reference to Table II reveals a fairly uniform average percentage of weight gains for the several groups both from the initial doses and after the doses were doubled. In the first series the order of percentage increases for the thirty-day period was: no medication, 47; physiologic saline, 59; methylglucamine, 49.5; glucophylline, 49. By the end of the first thirty days, then, the animals in all groups had made substantial weight gains, some of them better than 50 per cent. This, no doubt, accounts for the gradual tapering off of weight increases during the second phase of the experiment when the doses were doubled. Most of the animals had already reached maturity, after which weight increase was due primarily to deposition of fat and only secondarily to actual body growth. The total group average percentage gains were: no medication, 136; saline, 150; methylglucamine, 139; and glucophylline, 128.

TABLE II

WEIGHT CHANGES IN RABBITS

GROUP	ANIMAL	DOSE TOTAL	INITIAL WEIGHT (KG.)	WEIGHT IN 30 DAYS (KG.)	% INCREASE	DOSE TOTAL	WEIGHT ON FINAL DAY (KG.)	TOTAL INCREASE WEIGHT (KG.)	TOTAL % INCREASE
Control (a) No medication	1	0	0.88	Died					
	2	0	0.93	1.81	95	0	2.86	1.94	179
	3	0	0.98	1.74	78	0	2.61	1.64	168
	4	0	0.98	1.60	65	0	2.02	1.04	107
	5	0	1.05	1.66	57	0	2.59	1.54	146
	6	0	1.23	1.08	−12	0	2.20	0.97	80
(b) Physiologic saline	1	0.2 c.c.	1.23	1.82	49	0.4 c.c.	2.60	1.38	112
	2	0.2 c.c.	1.18	1.84	55	0.4 c.c.	2.55	1.38	116
	3	0.2 c.c.	1.15	1.76	53	0.4 c.c.	2.57	1.42	123
	4	0.2 c.c.	1.05	1.75	66	0.4 c.c.	2.60	1.55	147
	5	0.2 c.c.	0.95	1.58	66	0.4 c.c.	2.82	1.87	197
	6	0.2 c.c.	0.80	1.32	65	0.4 c.c.	2.48	1.68	209
Methylglucamine hydrochloride		mg./kg.				mg./kg.			
	1	8.3	1.50	2.08	39	11.6	3.22	1.72	115
	2	8.4	1.48	2.27	54	10.8	2.50	1.02	70
	3	9.0	1.38	2.02	47	12.3	2.60	1.22	89
	4	10.6	1.18	1.35	15	17.7	2.70	1.53	130
	5	10.8	1.15	1.79	55	13.9	2.74	1.59	140
	6	11.0	1.13	1.16	2	21.6	2.36	1.24	110
	7	11.5	1.08	1.33	23	19.1	2.33	1.26	110
	8	11.8	1.05	1.60	52	15.5	2.43	1.38	130
	9	12.1	1.03	1.50	46	16.5	1.92	0.89	81
	10	12.7	0.98	1.56	60	15.5	3.16	2.19	225
	11	13.1	0.95	1.54	62	16.4	2.57	1.62	170
	12	15.5	0.80	1.82	137	13.8	3.26	2.46	300
Glucophylline	1	11.5	1.75	2.24	21	17.9	3.56	1.81	103
	2	12.5	1.60	2.34	45	17.1	3.25	1.65	103
	3	14.3	1.40	2.05	45	19.5	2.82	1.62	100
	4	14.3	1.40	2.12	50	18.9	3.24	1.84	132
	5	16.1	1.24	1.95	57	20.5	2.95	1.71	137
	6	16.7	1.20	1.96	63	20.4	2.67	1.47	121
	7	16.7	1.20	1.82	50	21.9	2.76	1.56	130
	8	16.7	1.20	1.62	35	24.9	2.65	1.45	120
	9	17.4	1.15	1.62	42	24.9	2.49	1.34	116
	10	18.2	1.10	1.58	44	25.0	2.79	1.69	153
	11	18.2	1.10	1.78	62	22.2	2.93	1.83	166
	12	20.0	1.00	1.73	73	23.1	2.52	1.52	150

TABLE III

COMPOSITE BLOOD VALUES OF DOGS AND RABBITS

ANIMAL VALUES	R.B.C. MILLIONS	W.B.C. THOUSANDS	DIFFERENTIAL PERCENTAGE					HB. %	N.P.N. %	CREATININE %	NUMBER OF SAMPLINGS
			NEUT.	LYMPH.	MONO.	EOS.	BASOPH.				
Dog: "Normal values"*	7.2	11.8	69	20	6	5	0.7	77	30.8	1.5	?
Saline	5.5	6.7	71	25	4	4	0	111	32.4	1.4	21
M-glucamine	5.3	8.5	70	26	6	0	0	109	26.1	1.3	21
Glucophylline	5.4	10.0	77	21	2	0	0	103	28.9	1.5	20
Rabbit: "Normal values"*	5.6	7.9	43	42	9	2	4.0	95	31.0	1.1	
No medication	6.3	7.5	17	66	3	13	1	100	30.7	1.6	11
Saline	5.9	6.7	15	60	4	21	0	102	46.9	2.0	12
M-glucamine	5.5	8.1	10	68.5	5	16	0.5	104	38.1	2.0	20
Glucophylline	5.0	7.4	22	63.0	4	11	0	99	39.5	2.0	20

*"Normal values" of blood (count and chemistry) reported above are taken from Tables I and III, respectively, from Wagoner, G., and Custer, R. P.: *A Handbook of Experimental Pathology,* Springfield, Ill., 1932, Charles C Thomas.

2. Blood: Studies on blood changes included (1) erythrocytes, (2) white blood cells, (3) differential, (4) nonprotein nitrogen, and (5) creatinine. One complete weekly determination was made on each dog during the fifty-four days of the experimental period. In the case of the rabbits there was one complete weekly determination on each of eight animals, two within each group, the last being made at the time of disposition of the animal for histologic purposes. As with the dogs, the figures appearing in Table III represent the composite averages within each group. Included also in this table, for purposes of comparison, are figures of normal blood values for dogs and rabbits.*

3. Histologic: Tissues fixed in 10 per cent formalin were prepared for microscopic study from both sets of animals. The dogs were all disposed of on their last experimental day (the fifty-fourth); the rabbits were disposed in series of twos at ten-day intervals, beginning with the end of the first experimental period on the thirtieth day. Thus, whereas the first group that were treated had received a single daily dose for thirty days, the second, third and fourth groups received this, followed afterward by a double dose daily at intervals of ten, twenty, and thirty days, respectively. Although these animals were exposed to unequal quantities of the drugs over varying periods of time, no appreciable differences could be demonstrated in the tissues studied. Pulmonary hemorrhages, demonstrable in most samples studied, were due to ante-mortem traumatic injury. Samples of brain, lung, heart, liver, kidney, and spleen were examined (see Table IV).

From these studies it is apparent that glucophylline neither disturbs the growth nor checks the normal increase in the weight of immature rabbits. Furthermore, with respect to both dogs and rabbits no noticeable difference could be observed in the general appearance of treated and untreated animals regarding health and general state of nutrition. In these respects all the groups exhibited the same general condition of health. The microscopic study revealed the presence of cloudy swelling as a rather constant finding in samples taken from both dogs and rabbits. This finding was quite as conspicuous in the tissues of animals used as controls, as it was in those of animals treated with methylglucamine and glucophylline. The blood samples taken from our rabbits showed an inordinately high differential eosinophilic count. This phenomenon is a matter of not uncommon occurrence in the blood of laboratory animals. It is very probable that these cells, or many of them, were pseudo-eosinophiles or emphophiles. Referring to this type of cell, Wagoner and Custer[2] make the observation that "the analogue of the human neutrophile varies only in that the granules are larger than neutrophilic granules and take the acid stain by preference, although there is a slight affinity for the basic dye." Since this eosinophilic condition was uniformly present in each group, treated as well as untreated, we felt that we were dealing with cases of pseudo-eosinophilia and that these findings, therefore, were probably of no pathologic significance. Indeed, many a laboratory animal that appears to be normal and healthy in every respect has been found to exhibit visceral lesions exposed by the microscope, if not grossly visible at autopsy. In the course of these studies one of

*Taken from figures compiled by Wagoner and Custer and reproduced in Tables I and II, pages 33 and 34 of their *Handbook of Experimental Pathology*, Springfield, Ill., 1932, Charles C Thomas.

TABLE IV

TISSUE CHANGES IN DOGS AND RABBITS

SPEC. ANIMAL	BRAIN	LUNG	HEART	LIVER	KIDNEY	SPLEEN
A. Physiologic Salt Solution						
10. Dog 2	No change	No change	Slight cloudy swelling	Slight cloudy swelling	Slight cloudy swelling	Slight pigmentation. Slight increased cellularity in pulp
15. Rabbit A-4	No change	Hyperemia, small focal hemorrhages	Focal areas of necrosis with considerable inflammatory reaction	Cloudy swelling	Degeneration of tubular epithelium, especially in convoluted tubules	Slight hyperplasia
17. Rabbit A-3	No change	Slight hemorrhages	Cloudy swelling	Slight cloudy swelling	Hyperemia; cloudy swelling	Marked hyperemia
B. Methylglucamine						
7. Dog 4	No change	No change	Cloudy swelling	Marked cloudy swelling	Cloudy swelling	Hyperplasia
8. Dog 5	No change	Diffuse hemorrhages throughout	No changes	Cloudy swelling	Cloudy swelling, marked in some tubules; necrosis in some tubules	Hyperplasia
11. Rabbit B-1	--	Diffuse hemorrhages into alveoli, bronchi, and interstitial tissue	Marked hyperemia; cloudy swelling	Cloudy swelling	Hyperemia; cloudy swelling	
19. Rabbit B-8	No change	Small hemorrhages	Cloudy swelling	Cloudy swelling; increase in periportal connective tissue	Marked hyperemia; cloudy swelling	Hyperemia
13. Rabbit B-12	Slight thickening of meninges. Focal hemorrhage—focal area of softening with cellular reaction	Small hemorrhages; arteriosclerosis	Multiple hemorrhages; cloudy swelling	Cloudy swelling	Cloudy swelling. Leucocytic infiltration in interstitial tissue of cortex. Hyperemia	Hyperemia
5. Rabbit B-3	No change	Hyperemia; arteriosclerosis	Small hemorrhages. Slight increase in perivascular connective tissue	Cloudy swelling; passive hyperemia; maybe some fat	Cloudy swelling	Slight hyperplasia

TABLE IV—CONT'D

SPEC. ANIMAL	BRAIN	LUNG	HEART	LIVER	KIDNEY	SPLEEN
			C. Glucophylline			
9. Dog 9	No change	Hemorrhages in focal areas, cells with vacuolated cytoplasm in interstitial tissue. Suggest fat, slight fibrosis	Cloudy swelling	Cloudy swelling	Cloudy swelling	No change
12. Rabbit C-7	Hyperemia; infiltration of small and large monocytes in meninges	Small granuloma	Slight cloudy swelling	Cloudy swelling	Cloudy swelling; hyperemia; arteriosclerosis of small arteries	Slight hyperplasia
14. Rabbit C-2	No change	Diffuse hemorrhages	Cloudy swelling	Cloudy swelling	No change	Hyperplasia
18. Rabbit C-5	No change	Hyperemia, few small hemorrhages	Cloudy swelling; small hemorrhages	Cloudy swelling	Marked cloudy swelling; hyaline casts in collecting tubules	Slight hyperplasia

the female dogs in our kennels gave birth to a litter of six pups. On the day after birth we began giving daily injections of glucophylline to three of these pups, using the other three as controls. No significant differences have been noticed, and at the end of thirty days the relative individual weights have shown uniform increases.

SUMMARY

Laboratory studies on dogs and rabbits treated with methylglucamine and glucophylline revealed:

1. Growth of immature rabbits is not disturbed. These animals made increases in size and weight commensurate with normal controls.
2. The dogs, treated and controls, all lost weight—a condition due probably to food and housing restraint.
3. Histologic study of brain, heart, lung, spleen, kidney, and liver of both dogs and rabbits showed no harmful effects that might be attributed to the drug.
4. Significant figures setting forth results are appropriately tabulated.

We wish to express thanks to Dr. Robert S. Jason and Dr. Bernard Kapiloff for personal assistance, and, to the Abbott Laboratories for providing the drugs used in these experiments.

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CARCINOMA OF STOMACH DEVELOPING IN PERNICIOUS ANEMIA*

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THE combination of pernicious anemia and carcinoma of the stomach in one patient tends to rouse our curiosity as to the possible relationship between the two diseases. This inquisitiveness becomes more marked when one treats a patient for pernicious anemia and years later finds that that person has a carcinoma of the stomach. Many questions and possibilities arise. The following case is being reported as one that raises the discussion again. It is that of a patient who was treated for pernicious anemia for four and one-half years, at the end of which time an extensive carcinoma of the stomach was found. Table I shows the results of the various blood studies that were done during his course of treatments.

T. K., a 45-year-old Greek male, an unemployed kitchen helper, was admitted to the surgical service on Jan. 19, 1936, with a history of a mass in the right scrotum for some time. This suddenly became large and painful.

Examination revealed a tender scrotal mass, 7 by 6 by 5 inches, which transilluminated light and was moderately tender. There was discoloration of the distal third, the color being different from the remainder of the scrotum, suggesting to the surgeon that something recent had happened to it. Although the patient appeared pale, with a questionable lemon-yellow tint to the skin, immediate operation was decided upon. This was done under local anesthesia and a hydrocele with old and recent hemorrhages was found. The surgical condition cleared uneventfully.

The medical service then took over. The blood studies revealed a hyperchromic macrocytic anemia, as can be seen from the figures in the first line in Table I. The platelet count was normal. The red blood cell fragility test showed a slight increase in resistance, the figures ranging from 0.46 per cent sodium chloride to 0.28 per cent. The icteric index was 15. The sternal bone marrow was studied by Dr. Nathan Rosenthal, hematologist at the Mt. Sinai Hospital, who stated that the case was one of pernicious anemia. The Wassermann reaction was negative.

The gastric contents had no free hydrochloric acid on two occasions, histamine being used as a stimulant both times. They were of a heavy and mucoid character, with a total acid ranging from 7 to 12. No lactic acid was found. The benzidine test for blood was slightly positive on one occasion, but this was felt to be due to the trauma of passing the large stomach tube. Traces of urobilinogen were found in the urine. Stool examination did not show any occult blood, ova, or parasites.

The patient was treated with parenteral liver extract and a reticulocyte response of 12 per cent was obtained on the fifth to sixth day. It had varied from 0 to 1 per cent before the onset of treatment. A steady rise in the hemoglobin and red blood cell count took place. On discharge from the hospital on Feb. 26, 1936, the red cell count was 3,690,000 and the hemoglobin was 58.8 per cent. The cell volume index had dropped from 1.34 to 0.92. The icteric index had also dropped to 5.5 units.

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During his numerous visits to the clinic difficulties were encountered in trying to get cooperation, but these were mainly due to language differences. He was given biweekly, weekly, and monthly injections for the next four years, depending on the judgment of the physician who saw him in the medical clinic.

TABLE I

DATE	NUMBER OF RED BLOOD CORPUSCLES	PERCENTAGE HEMOGLOBIN	COLOR INDEX	MEAN CORPUSCULAR VOLUME	MEAN CORPUSCULAR HEMOGLOBIN	MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION
1/14/36	0.94	28	1.45	143	53.2	37.0
7/17/36	3.05	66	1.08	120	36.7	30.4
11/12/37	4.88	104	1.06	110.6	36.2	32.9
2/14/38	4.18	94	1.12	110	37.8	34.3
6/24/38	3.98	88	1.10	110.8	37.6	33.7
9/30/38	4.92	97	0.98	109.7	33.5	30.5
1/14/39	4.38	85	0.97	120	33	26
4/17/39	4.9	85	0.96	83	29	35
1/19/40	4.4	68	0.77	84	26	31

A neurologic examination was ordered as a routine procedure on Jan. 12, 1937, and the following findings were recorded: There was a loss of the pupillary light reflex. The biceps and triceps reflexes were diminished. There was a questionable diminution in vibratory sense along the shin bones and toes. A diagnosis of spinal cord disease, probably due to pernicious anemia, was made. However, a spinal fluid Wassermann was requested in order to rule out tabes dorsalis. This was done on Feb. 17, 1937, and the fluid showed a negative Wassermann and a normal colloidal gold curve. On Oct. 11, 1938, one and one-half years later, another neurologic examination was performed to determine the progress of the case, since liver extract had been given in the interim. This showed the pupils to react slightly to light. The biceps and triceps reflexes were still diminished. General sensory examination was negative. The conclusion was that there was probably no spinal cord disease.

The patient never had any complaints during his visits to the out-patient department. He suffered from the usual vicissitudes of existence in a slum, such as difficulty in obtaining extra food allowances. He was bitten by a dog in October, 1937, for which he was treated in the surgical out-patient department. In 1938 and 1939 he had all his upper teeth extracted and had to wait long for an upper dental plate. The plate proved to be a poor restoration and numerous repairs had to be made. During this period he had difficulty in eating a full diet and he lost some weight. His weight dropped from an average of 127½ pounds to 122½. However, in late 1939 and early 1940 his weight returned to its original figure.

In a two-week period in June, 1940, his weight dropped to 112 pounds, the lowest it had ever been. He was, therefore, watched closely, and by July his weight had decreased to 109 pounds. At this time some vague epigastric complaints were elicited by the physician in the medical clinic. In the gastrointestinal clinic he told the physician that he had had some epigastric pain for about two months. An x-ray of the stomach was ordered, and it indicated a large new growth in the middle portion of the stomach, on its greater curvature side (see Fig. 1).

At first he refused admission to the hospital but he later consented to enter. The weight at that time was 107½ pounds. Physical examination showed only emaciation and pallor. The blood showed hypochromic anemia, as seen by a red blood cell count of 2,880,000 and a hemoglobin of 40 per cent. A gastric analysis showed the free hydrochloric acid to be absent. Lactic acid was present. A four-plus reaction for blood was obtained. The stools were consistently positive for occult blood.

Exploratory laparotomy was advised in the hope that an operable lesion would be found. The patient refused this and left the hospital. The social service department kept an eye on him since he refused to return to the out-patient department for check-ups. He was admitted to Bellevue Hospital on Dec. 2, and died on Dec. 11, 1940.

AUTOPSY PROTOCOL

The autopsy protocol was furnished through the kindness and cooperation of Dr. Douglas Symmers, Director of Pathology, Bellevue Hospital:

The examination of the gastrointestinal tract reveals the lower one-third of the esophagus to be infiltrated with tumor tissue. The lumen is not constricted. The tumor tissue invades the mucosa and the underlying tissue of the esophageal wall. This tumor mass extends by direct continuity with the tumor growth seen in the stomach. There is no distention of the esophagus above the tumor site. The stomach is constricted in its midportion, resembling an "hourglass." On opening the stomach the lumen is found to contain a large amount of clotted blood. There is seen an ulcerating and fungating tumor mass which extends from the greater curvature over the entire posterior surface and around to the lesser margin of the stomach, leaving the anterior surface of the stomach free of tumor tissue.

Microscopic examination of the stomach tissue showed one part of the section to be replaced completely by tumor tissue. This tumor tissue extends from the mucosa, invades the underlying submucosa, muscularis, and serosa. Only small remnants of the aforementioned tissue are present. The tumor cells assume, for the most part, no set arrangement. Some, however, tend to assume a papillary form, and others, an acinar formation. The cells have no definite shape. They are fairly large cells with scant amount of eosinophilic cytoplasm and a large central oval vesicular nucleus with large nucleoli. Many mitotic figures are seen. Some of the tumor tissue has undergone necrosis.

The tail of the pancreas was found to be invaded for a short distance by the stomach tumor. Metastatic nodules were found in the liver, left adrenal, and surrounding lymph nodes. Microscopic examination showed this tissue to be similar to that in the stomach. No tumor cells are noted in the bone marrow. The different blood cells are present in their normal numbers.

The final diagnosis was as follows: Carcinoma of the gastrointestinal tract; fungating and ulcerating carcinoma of the stomach with hourglass constriction and hemorrhage into the lumen; extension into liver, pancreas, and esophagus; and metastases to mesenteric lymph nodes, liver, and left adrenal; moderate coronary atherosclerosis; mild atherosclerosis of the aorta; focal adhesive pleuritis; mild congestion of the liver; and general emaciation.

DISCUSSION

This case raised several interesting questions. One was whether or not this patient had had carcinoma of the stomach at the original discovery of his hyperchromic macrocytic anemia. The anemia was discovered accidentally when he was admitted to the hospital for a hemorrhage into a hydrocele. He had no complaints referable to his gastrointestinal tract. The only disconcerting fact was that he did not have an atrophic tongue. However, the various factors in the blood examination, such as color index, mean corpuscular volume, mean corpuscular hemoglobin, and bone marrow smears seemed to point to a diagnosis of pernicious anemia. The achlorhydria was a confirming factor, as were also the icteric index of 15 units and the response to liver treatment. The absence of occult blood in the stool at his first examination made us believe that there was no point in doing an x-ray of the gastrointestinal tract merely as a routine. This point, of course, cannot be settled in this patient. The type of carcinoma that was found, namely, a fungating, adenocarcinoma in the fundus of the stomach in the greater curvature side, has been called the "silent type" because it does not produce symptoms till very late in the course of the disease. It is still possible, therefore, that it might have been present all the time.

Some patients have been reported in the literature in whom gastric complaints have appeared during the course of pernicious anemia and in whom gastrointestinal x-rays were reported as showing no pathology. Subsequently, these patients developed carcinomas in various portions of the stomach. Conner and Birkeland[1] reported three such patients in whom carcinomas were found, respectively, in the esophagus and cardia of the stomach, in the pylorus, and in an unstated site in the third patient. Miller[2] reported two patients in whom carcinomas were found in the pylorus and in the middle portion of the greater

Fig. 1.

and lesser curvatures of the stomach. Of the seven patients reported by Washburn and Rozendaal[3] in whom a clinical diagnosis of pernicious anemia antedated the discovery of the carcinoma of the stomach, four patients had had previous x-rays of the stomach which showed no pathology. One patient had a scirrhous carcinoma of the upper two-thirds of the stomach; the second, a polypoid, ulcerating lesion removed by subtotal gastrectomy; the third, carcinoma of the distal half of the stomach; and the fourth, polypoid carcinoma involving the greater portion of the stomach.

Although no analysis has been made of the patients who had routine x-rays of the stomach, even though no gastrointestinal symptoms were present, it might seem advisable to perform such an examination on any patient who has the symptom complex, pernicious anemia. It, of course, might be advocated to examine any patient with pernicious anemia with a gastroscope, since lesions can be observed earlier with it than with an x-ray examination. However, the value of this procedure has not been demonstrated as yet in this type of case.

Of great interest in the case presented here is the change in the blood picture, which occurred some time before either loss of weight or gastric symptoms could be elicited. The figures in April, 1939, showed a marked drop as compared to the previous calculations. In January, 1940, this was even more marked with a hemoglobin of 68 per cent. At that time a search should have been made for some complications. Some authors[4, 5] have from time to time pointed out the necessity of searching for malignancies when the type of anemia in a treated case of pernicious anemia changes to a normocytic or microcytic form. Whether an operable carcinoma would have been found is a moot point. It is also impossible to state whether any lesion would have been discovered, although the size of the lesion found in July, 1940, makes me feel that some pathology would have been seen in January, 1940.

The literature records very few cases in which the blood count showed the suspicious changes before symptoms appeared. In the greatest majority of reported cases it was the appearance of new symptoms, or the inability to alleviate these complaints with liver therapy, which led to the x-ray studies of the gastrointestinal tract. One of the patients reported by Conner and Birkeland (Case 9)[1] had pernicious anemia for seven years when fresh blood was noted in a gastric analysis. Two x-rays taken two months apart were negative. A blood count done three months after the last x-ray examination, and following an attack of influenza, showed a hemoglobin of 29 per cent and an erythrocyte count of 2 million. Three months later he vomited blood, and an x-ray examination showed an obstructive lesion in the esophagus. Here the blood count changed to a low color index anemia before the x-rays disclosed a definite lesion. Numerous reports[1, 3-6] are present in the literature where a low color index anemia was discovered at the time that the carcinoma was found in the patients with pernicious anemia.

An interesting point and one which can lead to a great deal of speculation is to be found in the bone marrow studies. A bone marrow puncture, performed at the beginning of the observation, showed the cells to be typical of pernicious anemia. Nevertheless, study of the bone marrow at autopsy revealed no apparent changes.

CONCLUSIONS

A case of pernicious anemia is reported in which a carcinoma of the stomach was found four and one-half years after the initial diagnosis was made. Of particular interest is the fact that blood studies showed a change in the type of anemia several months before signs or symptoms of the carcinoma were found. It is suggested that blood studies be done repeatedly on patients with pernicious anemia and that a prompt search for pathology be made should a change in the character of the anemia be found.

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GUANIDINE AND ITS RELATIONSHIP TO MUSCULAR DYSTROPHY*

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THE abnormal metabolism of creatine (methylguanidine acetic acid) in progressive muscular dystrophy and certain other muscle conditions, as reported by Levene and Kristeller,[1] Brand and his co-workers,[2] Magee,[3] Thomas, Milhorat, and Techner,[4] Milhorat,[5] Tripoli and Beard,[6] and others, led me to study the effects of the administration of simple guanidine salts on creatine metabolism. It was first thought that administered guanidine might give rise to an added production and excretion of creatine. As the experiments progressed, the results suggested that the increased excretion of creatine following guanidine administration and in nutritional muscular dystrophy was due to changes produced in the muscle rather than to a direct production of creatine from the added guanidine.

MINIMUM LETHAL DOSE OF GUANIDINE HYDROCHLORIDE AND METHYLGUANIDINE SULFATE

Guanidine hydrochloride and methylguanidine sulfate were chosen for study. The minimum lethal doses of these compounds were determined to serve as a basis for their administration. The minimum lethal dose (M.L.D.) was defined as the number of milligrams of guanidine salt per gram of body weight which would cause the death within twenty-four hours of one or more of a series of fasting animals.

Guanidine hydrochloride and methylguanidine sulfate were administered intramuscularly and by stomach tube in a 5 per cent aqueous solution to healthy guinea pigs that had fasted twenty-four hours. The findings at the chosen end points are shown in Table I. Guinea pigs receiving the M.L.D. of either guanidine salt became nervous, showed varying degrees of trembling and twitching, and finally went into convulsions and died. Guinea pigs receiving less than the M.L.D. showed varying degrees of nervousness, trembling, and twitching, depending on the amount of guanidine salt given. Death occurred in some of the guinea pigs in this latter group, but later than twenty-four hours after the administration of the guanidine.

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PROTECTIVE ACTION OF GLYCINE

Schmeideberg and Bunge[7] were among the first to show that benzoic acid is detoxicated in the kidney by conjugation with glycine and then excreted as the less toxic hippuric acid. Since that time it has been found that this synthesis takes place in other parts of the body as well as the kidney. It also has been found that the animal organism protects itself in the same manner from many toxic substances, such as phenylacetic acid, cresols, and others. Perhaps this same mechanism comes into play in the detoxication of simple guanidine salts.

Single and daily doses of 1 mg. of glycine per gram of body weight were found to be nontoxic in healthy guinea pigs. The protective action of this amount of glycine was then studied.

TABLE I

FATALITY OF THE M.L.D. OF GUANIDINE SALTS

GUINEA PIGS USED	SALT ADMINISTERED	METHOD OF ADMINISTRATION	M.L.D. (MG. PER GM. OF BODY WEIGHT)	ANIMALS DIED IN 24 HOURS (PER CENT)
14	Guanidine hydrochloride	Intramuscular	0.24	60
13	Guanidine hydrochloride	Oral	0.40	80
10	Methylguanidine sulfate	Intramuscular	0.29	75
10	Methylguanidine sulfate	Oral	0.50	50

TABLE II

PROTECTIVE ACTION OF SINGLE DOSES OF GLYCINE AND GLUCOSE AGAINST GUANIDINE HYDROCHLORIDE

TOTAL NUMBER OF GUINEA PIGS	GUANIDINE HYDROCHLORIDE	GLYCINE	GLUCOSE	NUMBER OF GUINEA PIGS SURVIVING	PER CENT SURVIVING	AVERAGE BLOOD GLUCOSE IN SURVIVORS, 6 HR. AFTER GUANIDINE (MG. PER 100 C.C.)
11	M.L.D.	No	No	3	27	54
12	M.L.D.	Yes	No	9	75	75
8	M.L.D.	No	Yes	3	38	65

Single doses of 1 mg. of glycine per gram of body weight were given by stomach tube to healthy guinea pigs that had fasted twenty-four hours. One hour later, the M.L.D. of guanidine hydrochloride was administered intramuscularly to 6 of the guinea pigs. The M.L.D. of methylguanidine sulfate was administered intramuscularly to the remaining 6 guinea pigs. All the guinea pigs showed the usual symptoms of guanidine intoxication and the majority died within twenty-four hours. Glycine in single doses, therefore, has no protective action against the M.L.D. of toxic guanidine salts.

The protective action of daily doses of glycine was then studied. Twenty-four guinea pigs were divided into two groups. Group A received daily doses of 1 mg. of glycine per gram of body weight. Group B received no glycine. On the second and following days, one-third M.L.D. of guanidine hydrochloride was administered intramuscularly to 4 guinea pigs of each group, one-third M.L.D. of methylguanidine sulfate was administered intramuscularly to an-

other 4 guinea pigs of each group, and one-third M.L.D. of methylguanidine sulfate was administered orally to the remaining 4 guinea pigs of each group. The amount of guanidine salt in each dose was uniformly increased at regular intervals until four-fifths M.L.D. was being given daily.

The average life of the guinea pigs receiving only the guanidine salts was twenty-eight days; the average life of those receiving glycine in addition was thirty-five days. On long-continued administration of guanidine salts, the guinea pigs appeared to develop a greater tolerance to the drug. However, the guinea pigs receiving glycine showed fewer and milder symptoms of guanidine intoxication and in addition lived longer. It appeared, therefore, that administered glycine aided the animals in overcoming the effects of toxic guanidine salts.

PROTECTIVE ACTION OF GLUCOSE

Watanabe[8] and others found that a severe hypoglycemia developed after the administration of toxic guanidine salts. Frank, Nothmann, and Wagner[9] found that the severity of spasms and the duration of life of animals poisoned by guanidine were dependent upon the amount of carbohydrate available in their organisms. Hummel[10] also noted this protective action of glucose, but Bakucz[11] was not convinced of its effect.

Experiments similar to those above were performed to study the protective action of glucose. The effects of single doses of glucose and of glycine against the M.L.D. of guanidine hydrochloride are shown in Table II. In experiments with daily administration, the average life of the guinea pigs receiving guanidine hydrochloride alone was eighteen days; of the guinea pigs receiving guanidine hydrochloride and glucose, nineteen days; and of the guinea pigs receiving guanidine hydrochloride and glycine, twenty-seven days. Glucose appeared to have no protective action.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE URINE AND MUSCLE CHEMISTRY OF NORMAL GUINEA PIGS

The urine and muscles of guinea pigs were analyzed to serve as controls for comparison with the work to follow on dystrophic guinea pigs. Healthy guinea pigs were placed in small metabolism cages and given a diet of carrots, grain, water, and orange juice. The urine was collected under toluene, measured and filtered daily. The muscles were obtained at death or on sacrificing the animal.

Urine was collected for a ten-day period to serve as a normal. Treatment was then given as shown in Table III. One milligram of glycine per gram of body weight was administered daily at 10 A.M. and 4 P.M. One-third M.L.D. of guanidine hydrochloride was administered at 5 P.M. The amount of guanidine salt in each dose was uniformly increased at regular intervals, as in previous experiments, until four-fifths M.L.D. was being administered daily.

The urines were analyzed for creatine and creatinine,[12] guanidine,[13] and total acid-soluble phosphorus.[14] The results were averaged for the normal period and for the period in which treatment was given. The fresh gastroc-

nemius and gluteus muscles were analyzed for creatine,[15] creatinine,[12] and the acid-soluble phosphorus fractions.[14, 16, 17] The results were averaged and are shown in Table III.

TABLE III

URINE AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO NORMAL GUINEA PIGS

NO. OF GUINEA PIGS	4	4		4		4	
TREATMENT	NORMAL CONTROLS	NORMAL PERIOD	GLYCINE ADMINISTERED	NORMAL PERIOD	GUANIDINE ADMINISTERED	NORMAL PERIOD	GLYCINE AND GUANIDINE ADMINISTERED
Average days of treatment	42	10	32	10	26	10	32
Status at end of experiment	Alive		Alive		Dead		Dead
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)							
Creatine	1.5	1.5	1.9	1.6	1.8	1.6	2.1
Creatinine	2.9	3.0	3.0	3.1	3.0	3.1	3.1
Guanidine base	0.5	0.5	0.5	0.5	3.8	0.5	3.2
Total acid-soluble phosphorus	3.6	3.5	3.6	3.7	4.0	3.6	3.8
Per cent administered guanidine recovered	--	--	--	--	39	--	31
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)							
Creatine	533	--	597	--	406	--	500
Creatinine	4.7	--	4.8	--	4.2	--	4.4
Acid-soluble phosphorus							
Total	182	--	161	--	116	--	146
Creatine phosphate	69	--	66	--	28	--	48
(per cent of total P)	(38)	--	(41)	--	(24)	--	(33)
Free orthophosphate	23	--	19	--	24	--	30
(per cent of total P)	(12)	--	(12)	--	(21)	--	(20)
Adenosine triphosphate	74	--	64	--	49	--	52
(per cent of total P)	(41)	--	(40)	--	(42)	--	(36)
Soluble esters	16	--	12	--	15	--	16
(per cent of total P)	(9)	--	(7)	--	(13)	--	(11)
Water content (%)	76.8	--	76.1	--	75.8	--	76.3

On the administration of glycine alone to normal guinea pigs, the excretion of creatine was increased, probably due to an increased production. An increased concentration of creatine was found in the muscles, although the relative per cent of creatine phosphate changed but little from the normal.

On the administration of guanidine hydrochloride alone to normal guinea pigs, an average of 39 per cent of the guanidine hydrochloride was recovered from the urine. The per cent of guanidine bases excreted decreased as the guanidine hydrochloride administered was increased. There may have been a maximum amount of guanidine that the kidney was capable of excreting or the decreased excretion may have been due to kidney damage. Histologic section of the kidneys showed some damage to the tubules. Some of the guani-

dine may have been converted to creatine, as was suggested by the slight increase in the excretion of creatine. It was more probable, however, that the guanidine hydrochloride either injured the muscle cell or made it more permeable, allowing an excessive loss of creatine to occur, as shown by the low concentration of creatine in the muscle. This loss may have stimulated the muscle or the centers of creatine formation to a greater production of creatine, with a subsequent further loss due to the abnormal permeability of the cell. This suggestion of an increased permeability was further supported by the loss of phosphates from the muscle and the decrease in the relative per cent of creatine phosphate.

On the administration of glycine with the guanidine hydrochloride, the excretion of creatine was increased, the effect being greater than when either glycine or guanidine hydrochloride were given alone. An average of 31 per cent of the guanidine hydrochloride administered was recovered from the urine. This was less than where the guanidine hydrochloride was given alone, and indicated that the glycine probably aided in the detoxication of the guanidine hydrochloride. This detoxication prevented the profound action of the guanidine hydrochloride on the muscle, as shown by the improved muscle metabolism and by the analysis of the muscle. The creatine and total phosphorus of the muscle and the relative per cent of creatine phosphate more nearly approached the normal.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE URINE AND MUSCLE CHEMISTRY OF GUINEA PIGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

Goettsch and Pappenheimer[18] found that a diet composed of rolled oats, wheat bran, casein, lard, cod-liver oil, sodium chloride, and calcium carbonate, treated with ethereal ferric chloride, to which was then added skimmed milk powder, led to a progressive, highly selective and ultimately fatal dystrophy of the voluntary muscles. They found guinea pigs and rabbits to be susceptible and rats to be resistant. This diet is complete in known requirements except for vitamin E. However, Goettsch and Pappenheimer found that the addition of this factor did not prevent the development of the disease. The lesions were not due to inanition, infection, or scurvy, and must be referred to some still unknown factor.

A muscular dystrophy was developed in several litters of guinea pigs. Visible symptoms of muscular dystrophy were shown by the sluggish actions of the guinea pigs, difficulty in righting themselves when placed on their backs, inability to use their hind legs, and finally, inability to rise when placed on their sides. When definite symptoms of muscular dystrophy appeared, the weight had reached a plateau and in most cases was decreasing.

As previously noted, there occurs an abnormal metabolism of creatine in certain muscular dystrophies. Accordingly, the effects of glycine and toxic guanidine salts were studied in dystrophic guinea pigs.

Thirty-one dystrophic guinea pigs were divided into 4 groups. The dystrophy diet was continued. Group 1 was given no additional treatment. Group 2 was given 1 mg. of glycine per gram of body weight daily. Group 3 was given one-third M.L.D. of guanidine hydrochloride daily. Group 4 was given both

glycine and guanidine hydrochloride according to the amounts previously noted. The amount of guanidine salt in each dose was uniformly increased at regular intervals, as in previous experiments. The experiment was continued to the death of the guinea pigs. The chronologic data are shown in Table IV.

Twelve dystrophic guinea pigs were divided into 4 groups. They were placed in small metabolism cages, and the dystrophy diet and orange juice were continued. The urine was collected under toluene, measured and filtered daily. Urine was collected for seven days after dystrophy developed to serve as a control period. Treatment was then given these 4 groups as detailed for the preceding 4 groups.

TABLE IV

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ADMINISTERED TO GUINEA PIGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

TREATMENT	NO. OF GUINEA PIGS	AGE AT START OF DYSTROPHY (DAYS)	AGE AT TIME OF MARKED DYSTROPHY (DAYS)	INTERVAL FROM START TO MARKED DYSTROPHY (DAYS)	AGE AT DEATH (DAYS)	INTERVAL FROM START OF DYSTROPHY TO DEATH (DAYS)
None	7	94	103	9	110	16
Glycine	8	95	106	11	116	21
Guanidine hydrochloride	8	93	98	5	103	10
Glycine and Guanidine hydrochloride	8	97	105	8	112	15

The urines were analyzed as already detailed, and the results were averaged for each period of the experiment. The fresh gastrocnemius and gluteus muscles were analyzed as detailed. The results were averaged and are shown in Table V.

The guinea pigs in which a nutritional muscular dystrophy was developed excreted a larger amount of creatine than did normal guinea pigs. This may have been due to defective storage, to degeneration of the muscle tissue, or to a greater production. It is probable that all three mechanisms were involved. Dystrophic guinea pigs also excreted a smaller amount of creatinine as the dystrophy developed, probably due to the decreased creatine metabolism. Guanidine excretion was increased, probably due to defective metabolism of these compounds. The production of toxic guanidine compounds in the muscle cell may have lowered the permeability of the cell, thus accounting for the loss of creatine from the muscle and its increased excretion. An initial larger excretion of phosphorus was followed by a decreased excretion, indicating a probable loss of phosphorus compounds from the muscle. These findings were confirmed by the muscle analysis.

Glycine appeared to have some effect in delaying the progress of nutritional muscular dystrophy. Clinically, the animals appeared more normal and their span of life was increased. An increased excretion of creatine was noted and an increased concentration was found in the muscle. This increased concentration of creatine was responsible, probably, for the better functioning of the muscle and for the delay in the progress of the dystrophy.

The toxic effects of guanidine hydrochloride were more pronounced in dystrophic guinea pigs than in the normal. Glycine appeared again to reduce

its toxicity. On the administration of guanidine hydrochloride to dystrophic guinea pigs, an average of 63 per cent of the guanidine hydrochloride was recovered from the urine. These figures were markedly higher than those obtained with normal guinea pigs, indicating a defect in the metabolism and detoxication of these substances.

TABLE V

URINE AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO GUINEA PIGS WITH MUSCULAR DYSTROPHY

NO. OF GUINEA PIGS	3		3		3		3		12
TREATMENT	FIRST WEEK OF DYSTROPHY	BALANCE OF LIFE	FIRST WEEK OF DYSTROPHY	GLYCINE ADMINISTERED	FIRST WEEK OF DYSTROPHY	GUANIDINE ADMINISTERED	FIRST WEEK OF DYSTROPHY	GLYCINE AND GUANIDINE ADMINISTERED	AVERAGE OF ALL GUINEA PIGS FOR FIRST WEEK OF DYSTROPHY
Average days of treatment	7	9	7	14	7	5	7	11	7
Status at end of experiment		Dead		Dead		Dead		Dead	
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)									
Creatine	2.3	3.7	2.6	4.2	2.8	5.0	2.4	4.4	2.5
Creatinine	3.4	2.9	2.8	2.7	2.9	2.5	2.8	2.6	3.0
Guanidine base	0.5	0.6	0.6	0.6	0.5	3.9	0.6	4.8	0.6
Total acid-soluble phosphorus	3.7	3.0	3.5	3.1	3.0	2.3	3.1	2.9	3.3
Per cent administered guanidine recovered	–	–	–	–	–	63	–	53	–
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)									
Creatine	–	206	–	248	–	151	–	190	–
Creatinine	–	1.8	–	2.1	–	1.6	–	2.0	–
Acid-soluble phosphorus									
Total	–	76	–	88	–	47	–	66	–
Creatine phosphate	–	23	–	32	–	11	–	20	–
(per cent of total P)	–	(30)	–	(36)	–	(23)	–	(30)	–
Free orthophosphate	–	15	–	11	–	12	–	12	–
(per cent of total P)	–	(20)	–	(13)	–	(26)	–	(18)	–
Adenosine triphosphate	–	29	–	35	–	16	–	25	–
(per cent of total P)	–	(38)	–	(40)	–	(34)	–	(38)	–
Soluble esters	–	9	–	10	–	8	–	9	–
(per cent of total P)	–	(12)	–	(11)	–	(17)	–	(14)	–
Water content (%)	–	76.0	–	76.4	–	75.7	–	76.1	–

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE BLOOD, URINE, AND MUSCLE CHEMISTRY OF NORMAL DOGS

In the previous experiments the small size of the guinea pigs prevented extensive blood, urine, and muscle examinations. Dogs were adopted, therefore, as the test animal. Guanidine hydrochloride was used since it showed a greater toxicity than did methylguanidine sulfate. The compound was administered intramuscularly as it exerted a more profound effect in this manner.

The blood, urine, and muscles of 16 dogs were analyzed to serve as controls for comparison with the work to follow on dystrophic dogs. Healthy dogs were placed in metabolism cages and given the normal diet of meat and meal, with water ad lib. They were weighed twice a week. Urine was collected under toluene, measured and filtered daily. Blood was drawn twice a week. The muscles were obtained at death or on sacrificing the animal. Urine and blood were collected for a period of two weeks to serve as a normal. Treatment was then given as shown in Table VI.

One milligram of glycine per gram of body weight was administered daily by capsule. Eight-tenths milligram of guanidine hydrochloride per gram of body weight was injected intramuscularly one hour after the glycine. After three weeks the glycine and guanidine were increased to one and one-half times the original dose; after another two weeks, to two times the original dose; and after another two weeks, to two and one-half times the original dose. This latter dosage was continued to the death of the animals receiving guanidine hydrochloride.

The dogs receiving guanidine hydrochloride alone showed signs of guanidine intoxication after about the second week. They were nervous and showed trembling, which passed on to tetanic twitchings, convulsions, and death. The average life of these dogs was twenty-four days. The dogs receiving both glycine and guanidine hydrochloride were nervous and showed trembling and tetanic twitchings only two to three days before death. There were no other signs of intoxication over the period of the experiment. The average life of these dogs was forty-seven days.

The blood specimens were analyzed for sugar,[19] nonprotein nitrogen,[20, 21] creatine and creatinine,[22] amino acids,[23] guanidine,[24] inorganic and total acid-soluble phosphorus,[14] and lactic acid.[21, 25] The urines were analyzed for total nitrogen,[20, 21] creatine and creatinine,[12] guanidine,[13] and inorganic and total acid-soluble phosphorus.[14] The results were averaged for each period of the experiment. Table VI shows the average findings during the normal period and during the period of maximum dosage where the most striking changes were noted. Mixed samples of gastrocnemius, anterior tibialis, and biceps and quadriceps femoris were analyzed for creatine,[15] creatinine,[12] guanidine,[13, 26] acid-soluble phosphorus fractions,[14, 16, 17] sodium,[27, 28] potassium,[29] calcium,[27] chlorine by a modified silver nitrate and thiocyanate titration, and water content by drying to constant weight. The average results are shown in Table VI. Muscle and kidney specimens were examined histologically.

The muscles of the normal dogs and those receiving glycine were grossly and microscopically normal. The muscles of the dogs receiving guanidine hydrochloride alone showed many swollen and granular areas with a marked nuclear reaction. The muscles of the dogs receiving glycine and guanidine hydrochloride were normal grossly. Microscopically, a few swollen fibers were seen showing a marked nuclear reaction. The kidneys showed some damage to the tubules.

The oral administration of glycine to normal dogs produced an increased excretion of creatine. No other change was noted other than the increased blood amino acid concentration and an increased excretion of nitrogen which were to be expected.

TABLE VI

BLOOD, URINE, AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO NORMAL DOGS

NO. OF DOGS	4	4		4		4	
TREATMENT	NORMAL CONTROLS	NORMAL PERIOD	GLYCINE ADMINISTERED	NORMAL PERIOD	GUANIDINE ADMINISTERED	NORMAL PERIOD	GLYCINE AND GUANIDINE ADMINISTERED
Average days of treatment	62	14	48	14	24	14	47
Status at end of experiment	Alive		Alive		Dead		Dead
Average Blood Chemistry (mg. per 100 c.c.)							
Sugar	84	82	85	86	66	84	75
Nonprotein nitrogen	28	26	27	29	48	28	54
Creatine	3.1	3.1	3.3	3.1	4.5	3.1	3.8
Creatinine	1.4	1.4	1.8	1.3	2.6	1.6	2.6
Amino acids	7.9	7.6	9.4	8.2	6.8	7.7	9.5
Guanidine base	0.4	0.3	0.3	0.4	2.5	0.4	2.9
Inorganic phosphorus	7.0	7.2	6.6	6.0	6.3	7.6	6.4
Total acid-soluble phosphorus	36	41	36	30	34	40	34
Lactic acid	17	17	18	16	37	17	29
Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)							
Total nitrogen	38	36	48	39	45	37	46
Creatine	1.0	0.9	1.4	1.0	1.3	0.9	1.2
Creatinine	2.5	2.2	2.3	2.7	2.6	2.4	2.6
Guanidine base	0.6	0.6	0.5	0.6	3.3	0.6	2.8
Inorganic phosphorus	2.5	2.3	2.6	2.6	2.7	2.4	2.8
Total acid-soluble phosphorus	4.5	4.5	4.7	4.7	4.8	4.5	4.4
Per cent administered guanidine recovered	–	–	–	–	28	–	23
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)							
Creatine	428	–	431	–	313	–	406
Creatinine	4.9	–	5.1	–	4.2	–	5.1
Guanidine base	0.7	–	0.7	–	2.4	–	1.8
Acid-soluble phosphorus							
Total	214	–	225	–	187	–	209
Creatine phosphate	87	–	92	–	42	–	72
(% of total P)	(41)	–	(41)	–	(23)	–	(34)
Free orthophosphate	28	–	32	–	49	–	35
(% of total P)	(13)	–	(14)	–	(26)	–	(17)
Adenosine triphosphate	88	–	90	–	68	–	81
(% of total P)	(41)	–	(40)	–	(36)	–	(39)
Soluble esters	11	–	11	–	28	–	21
(% of total P)	(5)	–	(5)	–	(15)	–	(10)
Sodium	43	–	40	–	82	–	57
Potassium	319	–	306	–	271	–	285
Calcium	8	–	9	–	6	–	8
Chlorine	62	–	60	–	114	–	77
Water content (%)	77.7	–	77.5	–	76.2	–	77.1

The administration of guanidine hydrochloride to normal dogs produced an increased excretion of creatine. The greatest rate of excretion was found to occur in the first week or two of guanidine administration. It was probable, therefore, that the guanidine injured the muscle cell or lowered its permeability, allowing creatine to diffuse more rapidly than normal. The muscle was found to have a decreased creatine content, a decrease in the total acid-soluble phosphorus, and both a relative and an absolute decrease of creatine phosphate. A decrease in the blood sugar and an increase in the lactic acid showed a disturbance of the carbohydrate metabolism. An increased nitrogen retention was noted. A decrease in the potassium and an increase in the sodium and chlorine of the muscle showed a profound disturbance in the electrolyte balance. Guanidine bases were increased throughout, due to their administration.

The administration of glycine with the guanidine reduced the toxic action of the latter. The large supply of glycine aided directly in the detoxication and also promoted the production of creatine to replace that lost from the muscles. Metabolism in general appeared to be more normal. A nitrogen retention was still present, but less severe disturbances of carbohydrate metabolism and electrolyte balance were noted.

EFFECT OF GLYCINE AND GUANIDINE HYDROCHLORIDE ON THE BLOOD, URINE, AND MUSCLE CHEMISTRY OF DOGS WITH NUTRITIONAL MUSCULAR DYSTROPHY

A partial nutritional muscular dystrophy was developed in dogs. The dogs were weaned when they were forty-two days old and were placed in metabolism cages. The diet of Goettsch and Pappenheimer[18] was used. Sufficient water was added to the food to make a moist mass. This diet was supplemented by the use of additional skimmed milk powder dissolved in water, with the addition of vitamins A, C, and D. Water was allowed ad lib.

Morgulis and Spencer[30] found that the development of a nutritional muscular dystrophy following the diet of Goettsch and Pappenheimer could be prevented by the addition of whole wheat germ to the dystrophy-producing diet. Two dogs were placed on the diet with 10 per cent wheat germ added.

The dogs were weighed, and blood and urine were collected at two-week intervals during the first part of the experiment.

Visible symptoms of muscular dystrophy did not appear as rapidly nor was the dystrophy as pronounced as in the guinea pig. In about five months the weight had reached a plateau. Less interest was shown in food and the dogs appeared listless much of the time. A definite weakness finally appeared in the hind legs. Many of the animals died of pneumonia. These dogs were not included in the average findings given here.

When definite signs of weakness appeared in the hind legs, the dogs were weighed twice a week, urine was collected under toluene daily, blood was drawn twice a week, and the muscles were obtained at death or on sacrificing the animal. Treatment was then instituted as noted in Table VII.

The same amounts of glycine and guanidine were given as detailed above. After three weeks they were regularly increased as previously noted.

Blood and urine specimens were analyzed as previously discussed and the results were averaged for each period of the experiment. Table VII shows

TABLE VII

BLOOD, URINE, AND MUSCLE CHEMISTRY FOLLOWING THE ADMINISTRATION OF GLYCINE AND GUANIDINE HYDROCHLORIDE TO DOGS WITH MUSCULAR DYSTROPHY

NO. OF DOGS	4			2			2			2			2
TREATMENT	DYSTROPHY CONTROLS			GLYCINE ADDED			GUANIDINE ADDED			GLYCINE AND GUANIDINE ADDED			WHEAT GERM ADDED
	DYST. DIET	DYST. DIET	DYST. DIET	DYST. DIET	PLUS GLYCINE	PLUS GLYCINE	DYST. DIET	PLUS GUANIDINE	PLUS GUANIDINE	DYST. DIET	PLUS GLYCINE AND GUANIDINE	PLUS GLYCINE AND GUANIDINE	DYST. DIET PLUS 10% WHEAT GERM
Clinical State	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.	Sl. dyst.	Mkd. dyst.	Norm.
Average days of treatment	161	7	19	168	26	21	154	3	10	159	12	18	215
Status at end of experiment			Dead			Dead			Dead			Dead	Alive
Average Blood Chemistry (mg. per 100 c.c.)													
Sugar	78	75	60	76	85	73	73	61	54	76	87	73	85
Nonprotein nitrogen	25	28	43	27	33	42	30	41	49	28	32	47	27
Creatine	3.0	3.0	2.3	3.0	3.2	2.8	2.9	3.5	3.8	3.0	3.4	2.8	3.1
Creatinine	1.5	1.1	1.0	1.6	1.7	1.3	1.5	1.8	1.7	1.7	2.0	2.0	1.4
Amino acids	9.5	9.7	8.3	8.5	9.5	8.7	9.2	8.4	8.1	8.7	9.7	8.8	8.9
Guanidine base	0.4	0.6	0.6	0.4	0.5	0.5	0.4	2.2	2.6	0.4	0.7	1.4	0.3
Inorganic phosphorus	8.1	7.7	8.8	8.8	8.8	8.0	9.0	8.1	7.7	7.6	8.4	7.6	8.4
Total acid-soluble phosphorus	56	54	60	50	55	48	48	49	41	50	55	50	49
Lactic acid	19	21	17	19	20	19	20	32	34	18	21	28	18

Average Urine Chemistry (mg. excreted per 100 Gm. of body weight per day)													
Total nitrogen	94	88	95	95	105	110	84	92	97	90	100	103	84
Creatine	1.5	1.6	2.4	1.2	1.6	2.0	1.1	1.4	1.7	1.0	1.1	1.7	1.1
Creatinine	2.2	1.9	1.7	2.3	2.7	3.2	2.4	2.2	1.8	2.1	2.4	2.9	2.5
Guanidine base	0.7	0.8	0.9	0.6	0.6	0.7	0.8	1.8	2.5	0.5	1.0	2.5	0.6
Inorganic phosphorus	2.4	2.8	3.1	2.2	2.8	2.8	2.7	2.5	3.2	3.0	2.9	3.0	2.8
Total acid-soluble phosphorus	5.1	5.3	4.1	4.8	5.4	5.3	5.5	5.5	5.0	5.8	6.3	6.3	5.3
Per cent administered guanidine recovered	–	–	–	–	–	–	–	48	59	–	40	48	–
Average Muscle Chemistry (mg. per 100 Gm. of fresh tissue)													
Creatine	–	–	311	–	–	343	–	–	263	–	–	332	395
Creatinine	–	–	3.4	–	–	3.7	–	–	3.1	–	–	3.7	4.7
Guanidine base	–	–	1.1	–	–	0.9	–	–	2.9	–	–	1.4	0.6
Acid-soluble phosphorus													
Total	–	–	144	–	–	152	–	–	126	–	–	140	192
Creatine phosphate	–	–	46	–	–	58	–	–	31	–	–	45	78
(% of total P)	–	–	(32)	–	–	(38)	–	–	(25)	–	–	(32)	(41)
Free orthophosphate	–	–	27	–	–	20	–	–	33	–	–	29	23
(% of total P)	–	–	(19)	–	–	(13)	–	–	(26)	–	–	(21)	(12)
Adenosine triphosphate	–	–	55	–	–	59	–	–	43	–	–	51	81
(% of total P)	–	–	(38)	–	–	(39)	–	–	(34)	–	–	(36)	(42)
Soluble esters	–	–	16	–	–	15	–	–	19	–	–	15	10
(% of total P)	–	–	(11)	–	–	(10)	–	–	(15)	–	–	(11)	(5)
Sodium	–	–	90	–	–	61	–	–	112	–	–	87	45
Potassium	–	–	237	–	–	280	–	–	197	–	–	244	323
Calcium	–	–	32	–	–	15	–	–	18	–	–	23	9
Chlorine	–	–	117	–	–	101	–	–	122	–	–	112	71
Water content (%)	–	–	76.1	–	–	76.7	–	–	75.8	–	–	76.0	77.5

the average findings for the dogs in each group during the period when they were on the dystrophy diet alone and appeared to be normal clinically; during the period of slight dystrophy, as shown by slight weakness; and during the period of marked dystrophy, as shown by marked weakness before death. The muscle specimens were analyzed as already described, and histologic sections were made. The results of the chemical analyses are shown in Table VII.

At autopsy the muscles of the thighs and hind legs of the dogs, which showed marked weakness before death, were found to be pale in color and flabby. Areas of damaged fibers were seen. Many were swollen, showing degeneration and a marked nuclear reaction. A moderate infiltration with fibrous tissue was seen. The muscles of the dogs receiving 10 per cent wheat germ were normal.

Little metabolic change was noted until the muscular dystrophy became evident clinically. A slight increase in the excretion of creatine was noted, seen more especially in the daily analyses than in the averages shown. When marked muscular dystrophy developed, a further increase was noted in the excretion of creatine, accompanied by an increased excretion of total acid-soluble phosphorus. A low blood creatine concentration finally developed and a low concentration of creatine and creatine phosphate were found in the muscles. An increased concentration of guanidine base was found in the blood, urine, and muscle. The increased concentration of guanidine in the muscle may have produced an increased permeability, with the resulting loss of creatine and phosphate. The increased guanidine was due, probably, to some defect in the metabolism of guanidine compounds. A disturbance also was seen in the carbohydrate and mineral metabolism, similar to that seen in the normal dogs when toxic guanidine compounds were injected intramuscularly.

Glycine appeared to delay the effects of the nutritional muscular dystrophy, as shown by the blood, urine, and muscle chemistry. The animals lived longer and their metabolism was more normal. The basis of this improvement may have been the increased concentration of creatine in the muscle due to its increased production.

Guanidine hydrochloride showed a greater toxicity in dogs suffering from a nutritional muscular dystrophy than it did in normal dogs. Glycine again aided in the detoxication of this added guanidine.

DISCUSSION

The administration of toxic guanidine salts over a period of several weeks results in a loss of creatine and phosphate from voluntary muscle. It appears probable that guanidine increases the permeability of the muscle cell, allowing creatine and phosphate to diffuse more rapidly than normal when they have been separated during muscular action. This suggestion is supported by the work of Gavrilescu,[31] who found by conductivity experiments that guanidine compounds increased the permeability of abdominal muscles. The lowered concentration of creatine in the muscle may stimulate the centers of creatine production, resulting in an increased production and further loss of creatine.

Increased blood guanidine and increased guanidine excretion have been noted in many conditions. Much confusion has resulted from the attempt to

associate various clinical symptoms with the level of blood guanidine. It is suggested that blood guanidine is the result, and not the cause, of endogenous guanidine intoxication. A guanidine derivative produced in any tissue may exert a more profound action in that tissue than it may exert after diffusion into the extracellular body fluids. The level of the blood guanidine probably depends on the activity of the centers of detoxication.

In exogenous guanidine intoxication other physiologic effects of guanidine may overshadow its action on the muscle cell. This effect is increased by the apparent ability of the animal to acclimate itself to larger amounts of administered guanidine over a period of time.

There is a similarity in the blood, urine, and muscle chemistry of guanidine intoxication and of nutritional muscular dystrophy. The increased concentration of simple guanidine compounds in nutritional muscular dystrophy suggests the possibility that these compounds may be the cause of the metabolic disturbance in this condition. The increased concentration of guanidine in the blood and urine of patients with progressive muscular dystrophy, as found by MacFate,[32] Sullivan, Hess, and Irrevere[33] and others, and the similarity of the muscle findings in this paper to the findings in progressive muscular dystrophy, as reported by Nevin,[17] Reinhold and Kingsley,[34] and others, suggest the possibility that toxic guanidine compounds may be responsible for, or at least initiate, the abnormal processes found in progressive muscular dystrophy and other dystrophies accompanied by like metabolic changes.

Hines and Knowlton[35] believe that the findings of altered concentration of various substances in atrophic muscles can be attributed to relative changes in the amounts of muscle cell and nonmuscle cell phases rather than to a change in the composition of the various phases. They found the increase in chloride concentration of atrophied muscle (from denervation, fasting, and tenotomy) to parallel the extent of atrophy and the relative increase in connective tissue, and, therefore, concluded that the chlorides of the muscle are chiefly associated with the connective tissue. Morgulis and Osheroff[36] state that at least a part of the chloride is a component of the connective tissues and that the increase in chloride content of dystrophic muscles is undoubtedly associated with the increase in fibrous tissue. The moderate increase in connective tissue noted in these experiments may account, in some degree, for the chemical changes observed. However, microscopic study did not reveal as great an increase in the connective tissue as is usually seen in cases of atrophy from denervation, fasting, and tenotomy. Further, because of the rapid and early chemical changes noted, it is believed that some other factor, such as an increased permeability of the muscle cell, must be considered.

The therapeutic value of glycine in these conditions probably lies in its stimulation of an increased production of creatine. This may be due to the specific dynamic action of the glycine itself (Rapport and Katz[37]). An increased glycine concentration in the blood and body fluids may stimulate the production of large amounts of creatine until the creatine production centers adjust themselves to this new and increased level of glycine. The rate of creatine production may then drop to approximately normal, accounting for the decreased excretion of creatine found in many patients after glycine therapy

over a long period of time, as reported by Thomas, Milhorat, and Techner,[4] Milhorat,[5] and others. The value of glycine in the treatment of progressive muscular dystrophy apparently depends upon the ability of the muscle cell to utilize the creatine produced. This ability appears to be lost gradually as the muscular dystrophy progresses.

The toxicity of guanidine derivatives varies greatly (Alles[38]). The general finding of increased blood guanidine cannot be properly evaluated until methods are perfected for determining the exact guanidine derivative present.

CONCLUSIONS

1. The daily administration of one-third to four-fifths M.L.D. of a toxic guanidine salt, over a period of several weeks, produces changes in the metabolism and structure of the muscle, similar to those found in nutritional muscular dystrophy and in progressive muscular dystrophy. There are produced a loss of creatine and phosphate from the muscle and severe disturbances in carbohydrate metabolism and the electrolyte balance. It is suggested that guanidine may lower the permeability of the muscle cell, producing these changes.

2. One milligram of glycine per gram of body weight, administered daily, appears to afford some protection against small daily doses (one-third to four-fifths M.L.D.) of a toxic guanidine salt.

3. Glycine delays the progress of a nutritional muscular dystrophy.

4. It is suggested that the abnormal metabolism of guanidine compounds may be an etiologic factor in the production of a progressive muscular dystrophy.

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A COMPARATIVE STUDY OF DEXTROSE AND DEXTRIN TOLERANCE IN PATIENTS WITH CHRONIC ULCERATIVE COLITIS*

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ABSORPTION studies in chronic ulcerative colitis have been confined mainly to dextrose, and it seemed of interest to us to study the intestinal absorption and the blood sugar response of another carbohydrate. For this study, a mixture of polysaccharides (dextrins and maltose) produced by the partial breakdown of starch was selected. For control purposes dextrose absorption was studied simultaneously. Tolerance curves served as a measure of the absorption process.

Low or flat blood sugar curves for oral dextrose tolerance tests have been frequently found by us (Z. B. and R. C. P.)[1] in patients with chronic ulcerative colitis. In one group of 58 standard oral, one dose, two-hour dextrose tolerance tests, 23 (39.6 per cent) tests were of the low or flat type. Groen,[2] using a simplified technique of intestinal intubation, ascribed the decreased absorption of dextrose to a defect in the absorptive capacity of the intestinal wall and not to an unusually rapid passage of the intestinal contents.

METHOD OF STUDY

Twenty-three patients with chronic ulcerative colitis attending an outpatient clinic were studied. All the patients had had the disease for periods of one or more years.

Both the oral dextrose tolerance test and the oral dextrin tolerance test were carried out on the same patient. These tests were alternated in successive patients. The tests were carried out from six to ten days of each other, with no change in diet, so that each patient served as his own control.

The patients reported in the morning without breakfast. They were weighed and a fasting venous blood sample was obtained. One gram per kilogram of body weight of dextrose or dextrin† in 150 to 200 c.c. water was given, and blood samples were taken at one-, two- and three-hour intervals, and in some instances at four and five hours. Urine was also collected and examined for dextrose at these intervals. The venous blood was analyzed for true dextrose content by the Shaffer-Somogyi[3] method. The results, therefore,

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†Since pure dextrin was found unsuitable because of unpalatability and difficulty of making acceptable solutions, "Dexin" (Burroughs Wellcome & Co.), containing 75 per cent dextrins and 24 per cent maltose, 0.75 per cent moisture, and 0.25 per cent mineral matter, was used. Since the predominant constituent of this polysaccharide and disaccharide combination consists of dextrin, we have used the term *dextrin* in the text to denote this dextrin-maltose preparation.

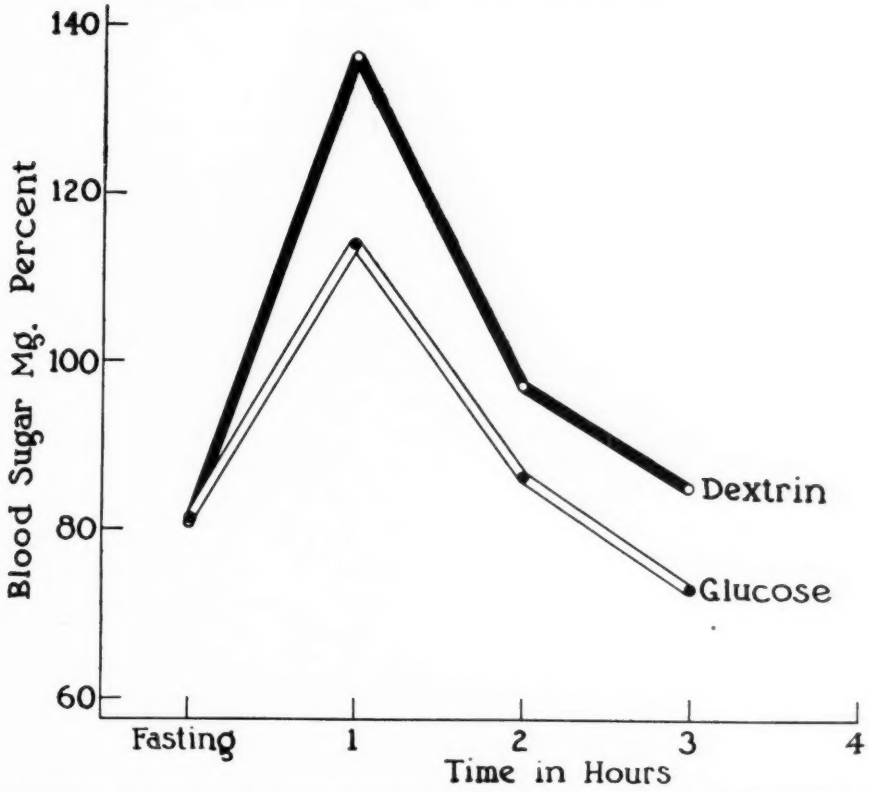

Fig. 1.—Graph showing mean values of comparative results of oral dextrose and dextrin tolerance tests on 23 patients with chronic ulcerative colitis, as given in Table I.

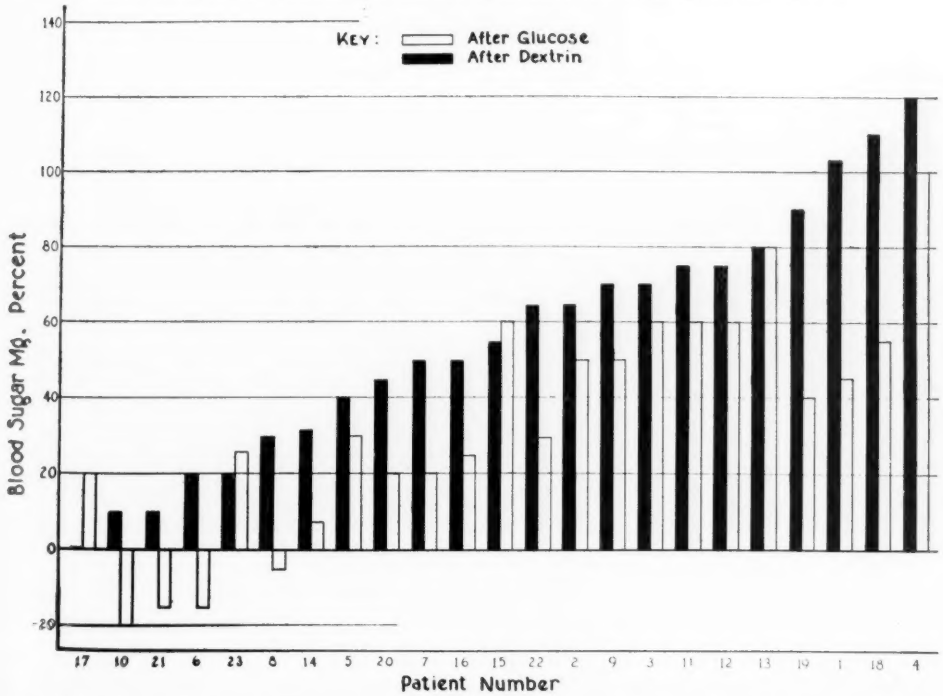

Fig. 2.—Bar graph showing comparison of increase or decrease in blood dextrose level from fasting level one hour after oral dextrose and dextrin tolerance tests in the same patients.

represent dextrose content with only little interference from other reducing substances (vitamin C, glutathione, methionine, cysteine, etc.).

RESULTS OF STUDY

The results are given in Table I and are presented graphically in Figs. 1 and 2. It will be observed that after dextrose administration the blood sugar at the end of the first hour had risen sharply to an average value of 115 mg. per 100 c.c., an increase of approximately 42 per cent over the average fasting level of 81 mg. per 100 c.c. However, at the end of the second hour it had dropped sharply to 86, being then only 7 per cent above the fasting level. At the end of the third hour it had fallen to 73, or 10 per cent below the fasting level. At the end of the fourth hour the lowest value was reached, 69, or 15 per cent below the fasting level. The four determinations available at the fifth hour showed that blood sugar was starting to recover, now being 75, or 7 per cent below the fasting level.

TABLE I

PATIENT NO.	GLUCOSE						DEXTRIN					
	TRUE BLOOD GLUCOSE (MILLIGRAMS PER 100 C.C.)—SHAFFER-SOMOGYI METHOD											
	FASTING LEVEL	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	FASTING LEVEL	1 HR.	2 HR.	3 HR.	4 HR.	5 HR.
1	70	115	110	85	80		105	208	190	135	110	
2	80	130	95	90	80	85	95	160	145	95	65	80
3	90	150	140	95	80	95	70	140	145	105	75	60
4	80	180	110	70			80	200	135	70		
5	85	115	100	55	55		70	110	65	60		
6	95	80	75	80			80	100	85	90		
7	65	85	60	90			60	110	100	90		
8	105	100	95	60			110	140	105	85		
9	80	130	135	115	85		80	150	160	100		
10	90	70	45	70			70	80	60	60		
11	70	130	70	60			85	160	80	70		
12	95	155	95	80			75	150	70	60		
13	70	150	75	60	70	75	70	150	85	65	65	
14	90	98	65	75	80		80	113	75	75		
15	70	130	65	60	60		80	135	85	75		
16	105	130	80	70			85	135	90	65		
17	65	85	55	45	55		70	70	75	75	60	
18	80	135	100	50			70	180	105	50		
19	55	95	80	45	45	45	65	155	85	140		
20	80	100	95	90	65		115	160	115	95	85	
21	100	85	60	85			80	90	50	85		
22	70	100	90	70			80	145	125	130		
23	70	95	90	80	70		75	95	105	85	75	
Means	80.9	114.9	86.3	73.0	68.8	75.0	80.4	136.4	97.2	85.2	76.4	70.0

After dextrin administration the blood sugar showed a similar but much greater rise by the end of the first hour. From the fasting level of 80 mg. per 100 c.c., the blood sugar rose to 136 at the end of the first hour, an increase of 70 per cent. By the end of the second hour the drop had not reached as low a level as with dextrose, the figure being 97, or 21 per cent above fasting. At the end of the third hour the blood sugar was 85, or still 6 per cent above the fasting level. The seven available observations at the end of the fourth hour gave 76.4, or a drop of 5 per cent below fasting level. The two fifth-hour observations averaged 70, or 13 per cent below fasting level.

Urinalyses on the entire group of patients using quantitative Benedict's test revealed reducing substances in only one patient (No. 8). The fasting specimen was negative, and in the first-hour specimen after oral dextrose the reducing substances were present to the amount of 1.65 Gm., after the second hour 1.13 Gm., and after the third hour 0.45 Gm. In the dextrin test the fasting specimen was negative. After one hour the urine contained 0.59 Gm. of reducing substance, after the second hour 0.45 Gm., and after the third hour 0.19 Gm.

DISCUSSION OF RESULTS

The foregoing observations apparently indicate that at all points the blood sugar levels were higher after the dextrins than after the dextrose. However, it is well known that because of the many variables involved, blood sugar curves undergo wide fluctuations. Therefore, a statistical analysis of the results was made in order to determine whether the greater hyperglycemia following the dextrin administration was real or whether it was only an accident due to chance.

Statistical Analysis.—The dextrin and the dextrose curves were compared by calculating the statistic "t" for the differences in the blood sugar means at the one-hour, two-hour, and three-hour periods. The respective "t" values found were 5.568, 3.846, and 2.756, and the corresponding probabilities, as given by the tables of Fisher and Yates,[4] were $P = <0.001$, $P = <0.001$ and $P = >0.001$ but <0.01. Since a probability of $P = 0.001$ indicates one chance in 1,000 and a probability of $P = 0.01$ indicates one chance in 100, one may conclude the difference is real and that dextrin administration produced higher blood sugar values than did dextrose administration.

Since 100 mg. of the dextrin mixture are theoretically capable of giving approximately 108 mg. of dextrose if completely hydrolyzed, an approximate correction was made by reducing the mean difference by 8 per cent and again calculating the "t" values. This procedure did not appreciably alter the results.

One hundred milligrams of the dextrin preparation used in this study contained 75 mg. of dextrin, $(C_6H_{10}O_5)_n \cdot H_2O$, which if completely hydrolyzed would yield 83.3 mg. of dextrose (using the factor of 0.9, since nine parts of dextrin give ten parts of dextrose upon hydrolysis),[5] and 24 mg. of anhydrous maltose ($C_{12}H_{22}O_{11}$), which would yield 25.2 mg. of dextrose (using the factor of 0.95) if completely hydrolyzed, making a total of 108.5 mg. of dextrose. Actually the maltose is present in the hydrated state ($C_{12}H_{22}O_{11} \cdot H_2O$) so that 24 mg., when hydrolyzed, yields 24 mg. of dextrose (1.2 mg. less than with anhydrous maltose). Using this figure in the calculation, 100 mg. of the dextrin-maltose mixture would yield 107.3 mg. of dextrose instead of 108.5 mg. We have compromised between these two values and assume that 100 mg. of the dextrin mixture would be converted into 108 mg. of dextrose, or an 8 per cent increase over the original weight.

The possibility exists that a comparison of the areas under the blood dextrose curves may present a more comprehensive picture than comparisons made separately at three different points. Accordingly, the areas under the 46 curves were measured with a polar planimeter. Areas below a base line passing through the fasting level were considered negative. These data were subjected to an analysis of variance following the standard procedure, such

as outlined by Bliss and Marks.[6] The variance ascribable to differences in patients was about 25 per cent of the total. This points to the necessity of using a considerable number of patients and emphasizes the wisdom of having each patient serve as his own control. The "t" value of 6.305 ($P = <0.001$) again shows that dextrins were significantly more effective in raising the blood sugar level than was dextrose.

It is impossible to state with certainty the reason for dextrin causing a higher and more prolonged rise in the blood sugar level than with dextrose itself. It is generally held that dextrins in the intestine undergo enzymic hydrolysis to maltose and then to dextrose, which is absorbed in the usual manner. The time factor of this hydrolysis may be the reason for the prolonged rise of blood dextrose after dextrin. Two other possibilities also present themselves: better absorption of the dextrose derived from dextrin, or a slower storage of the dextrose as glycogen by liver and muscle, although no evidence exists as to why such dextrose should behave different from pure dextrose. It is also recognized that the dextrins may on hydrolysis yield some slowly metabolized reducing substance which could react with the Shaffer-Somogyi reagents. No information is available on this point.

SUMMARY AND CONCLUSIONS

1. Twenty-three patients with chronic ulcerative colitis were studied as to the oral dextrose and oral dextrin tolerance tests.

2. One hour after dextrose administration the true blood dextrose had risen 42 per cent over the average fasting level while after the dextrin administration the blood dextrose had in the same time increased 70 per cent.

3. Two hours after dextrose the blood dextrose level was 7 per cent above the fasting level while the level in the dextrin group was 21 per cent above the fasting level.

4. Three hours after dextrose the blood dextrose level was 10 per cent below the fasting level while in the dextrin group it was 6 per cent above the fasting level.

5. A dextrin preparation maintained significantly higher blood dextrose levels for three hours than did dextrose in a group of patients with chronic ulcerative colitis. These findings have led to the assumption that in the patients studied dextrin was better absorbed from the intestine than was dextrose.

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HYPERBILIRUBINEMIA FOLLOWING ADMINISTRATION OF SULFONAMIDES*

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THE present report is concerned with the occurrence of hyperbilirubinemia and other evidence of hepatic functional impairment during or after administration of sulfonamide compounds to twenty patients.

COMMENT

The pertinent data are presented in the table. Visible icterus was absent in six instances (Cases 1, 4, 12, 14, 15, 19). Total and direct-reacting bilirubin was determined by the method of Malloy and Evelyn.[1] The existence of hyperbilirubinemia was first noted one to seven days after institution of sulfonamide therapy, after administration of 6 to 24 Gm. of the drug, and with blood sulfonamide concentrations of 4 to 14 mg. per 100 c.c. Recovery occurred in every case.

It is generally stated that hepatocellular damage occurs not infrequently during administration of sulfanilamide,[2-9] seldom with sulfapyridine,[2] and rarely with sulfathiazole,[2] while it has not been observed following the use of sulfadiazine.[2, 10] In the present series, evidence of hepatic functional impairment, other than hyperbilirubinemia and excessive urobilinuria, was demonstrated in Cases 2, 6 (sulfanilamide), 10, 11, 13 (sulfathiazole), 15, 16 (sulfapyridine), 17, 18, and 20 (sulfadiazine). In Case 7 the hyperbilirubinemia was probably fundamentally of hemolytic origin, as suggested by the absence of bromsulfalein retention, the anemia, and the relatively low proportion of direct-reacting bilirubin in the serum. The same may be true of Cases 4 and 19. In Cases 1 and 2, 4 Gm. of sulfanilamide was placed in the peritoneum at the time of operation. It has been shown[6] that additional sulfonamide therapy following this procedure enhances the possibility of development of hepatitis. Because of the occasional development of hyperbilirubinemia and other evidence of hepatic functional impairment in patients with peritonitis and pneumococcus pneumonia, their occurrence in Cases 1, 3, 12, 19, and 20 cannot be attributed definitely to the effect of the sulfonamides.

CONCLUSIONS

These data indicate that hyperbilirubinemia and other evidence of hepatocellular damage may occur during the course of administration of sulfadiazine, as well as sulfathiazole, sulfapyridine, and sulfanilamide. Routine determina-

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TABLE I

CASE	CONDITION	SULFONAMIDE	DOSE (GM.)	DAYS	BLOOD SULFONAMIDE (MG. PER 100 C.C.)	SERUM BILIRUBIN		BROMSULFALEIN RETENTION* (%)	UROBILINOGEN IN URINE (URINE DILUTION)	HIPPURIC ACID EXCRETION† (GM.)	OTHER URINE FINDINGS	HB. %	ERYTHROCYTES (IN MILLIONS)	LEUCOCYTES (IN THOUSANDS)
						TOTAL (MG. PER 100 C.C.)	DIRECT (MG. PER 100 C.C.)							
1	Salpingitis	Sulfanilamide	8#	5	8.0	1.1	0.66					(78)	(4.2)	(14.6)‡
	Pelvic peritonitis		12	9	7.2	1.5	0.8		1:100			55 54	3.8 2.9	15.2 2.9
2	Cecostomy	Sulfanilamide	8#	2	6.5									
				4	8.2	1.1	0.5					(80)	(4.3)	(16.0)
			32	8		3.8	2.5					110	4.1	11.2
				9	7.8	5.0	3.1	30	1:800		Bilirubin	78	3.4	12.0
				10	2.8	5.0	3.1							
				14		3.0	1.7	10	1:20		Bilirubin	71	3.9	10.7
				28		0.4		0	1:20					
3	Pelvic abscess	Sulfanilamide	24	3	4.0	5.3	4.0					(80)	(4.0)	(8.2)
				8	0.0	10.5	7.3					75	4.0	8.9
4	Tonsillitis	Sulfanilamide	18	3	4.3	1.2	0.2		1:200					18.0
5	Acute appendicitis	Sulfanilamide	28	3	7.3	3.0	1.4		1:200		Blood, albumin casts	(82) 84	(4.2) 4.2	(16.9) 10.0
6	Acute appendicitis	Sulfanilamide	16	2	4.4	3.7	0.7		1:50	1.1	Bilirubin	90	4.8	19.6
			24	5		2.5	1.0		1:100		Bilirubin	97	4.8	26.4
7	Gonococcus urethritis	Sulfanilamide	?	1	6.3	5.9	0.5					52	2.8	4.2
				3		4.6	0.5	0	1:300					
				4	2.1	2.9	0.7							
				5		1.6	0.8		1:500			58	3.0	5.7
				6		1.1	0.5							
				10	0.2	1.0	0.5							
				12	0.0	0.4	0.2		1:40			62	3.5	5.4
8	Acute appendicitis	Sulfathiazole	17.6	6	14.0	1.8	1.0					(65) 60	(3.5) 3.8	(25.6) 19.7
9	Acute appendicitis	Sulfathiazole	10	2	8.6	2.0	1.2		1:400					
10	Gonococcus urethri-	Sulfathiazole	?	4	7.3	1.4	0.9		1:200		Albumin casts	80	4.2	6.3
				5	5.2	1.7	1.0	10	1:100					
				6	2.4	2.7	1.7	20	1:100	1:5				

10	Gonococcus urethritis	Sulfathiazole	?	4 5 6	7.3 5.2 2.4	1.4 1.7 2.7	0.9 1.0 1.7	 10 20	1:200 1:100 1:100	 1:5	Albumin casts	80	4.2	6.3
11	Gonococcus urethritis	Sulfathiazole	?	5	9.2	2.5	1.2	10						
12	Pelvic peritonitis	Sulfathiazole	20	3	6.2	1.6	0.7							
13	Pelvic peritonitis	Sulfathiazole	20	3	5.6	6.6	2.5	15	1:50			72	4.0	10.6
14	Gonococcus urethritis	Sulfathiazole	10	2	8.0	1.0	0.5		1:400					
15	Gonococcus urethritis	Sulfapyridine	12	2 7 13	4.2 0.0 0.0	1.5 1.5 0.5	1.0 0.8	15 15 0	1:50 1:50 1:20	 1.6 3.0	Blood crystals	90	4.6	7.9
16	Pyelonephritis	Sulfapyridine	40	7 8 12 35 39	9.1 6.8 0.5 0.0 0.0	2.0 5.4 5.9 1.8 1.0	0.8 3.0 3.0 1.0 0.7	 40		 1.6		(77) 68 51 89	(4.0) 3.7 2.8 4.6	(12.0) 10.3 18.0 13.2
17	Pyelonephritis	Sulfadiazine	12 18	2 3 6 8	6.2 9.3 2.0 0.5	2.1 3.2 1.8 0.8	1.4 2.0 1.3	20 30 10	1:100 1:100 1:50	 1.1		(82) 80	(4.5) 3.9	(12.6) 9.2
18	Pyelonephritis	Sulfadiazine	18	3 4 7 12	7.1 7.0 5.3 0.6	1.7 2.2 2.1 1.2	1.3 1.6 1.6 1.0	30 30 20	1:200 1:100 1:50	 1.3		(72) 68	(3.7) 3.5	(9.8) 8.4
19	Pneumococcus pneumonia	Sulfadiazine	18	3 4	9.6 6.8	1.5 1.3	0.6 0.5	 0	1:200		Albumin casts	82	4.5	19.0
20	Pneumococcus pneumonia	Sulfadiazine	6	1 2 5 6	4.7 11.7 4.4	4.2 2.6 1.3 1.0	3.0 1.9 0.7 0.5	 30	 1:100	 1.9		(85) 60	(3.9) 3.5	(25.0) 22.0
			22	8	6.6							82	4.5	10.0

*Retention in blood thirty minutes after 2 mg. per kilogram.
†Excretion four hours after 6 Gm. of sodium benzoate by mouth.
‡Figures in parentheses values before sulfonamide therapy.

tions of serum bilirubin concentration and tests of hepatic function would probably reveal a much higher incidence of hepatic functional impairment than has been reported heretofore following use of these agents.

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LABORATORY METHODS

GENERAL

A STUDY OF THE PHENOMENON OF ERYTHROCYTE SEDIMENTATION*

IV. Venous Stasis, Delay, Temperature of Sample, Containers, Agitation, and Quantity of Formed Elements as Sources of Technical Variations

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IN PREVIOUS publications a critical survey of considerable literature,[1] choice of a reliable technique for the measurement of sedimentation,[2] and evidence substantiating my choice of the kind and quantity of anticoagulant[3] were presented. It is the purpose of this paper to discuss additional sources of technical variation and evidence of their control.

Free flow of blood without venous stasis cannot be accomplished in the horse unless a large enough needle is employed—at least a No. 10 gauge needle, or better yet, a fair-sized cannula. Repeated bleeding with a needle of this size creates considerable damage and is not advised. In lieu of the use of a large needle, a sample can be taken with a syringe (50 c.c. glass) and a smaller needle (No. 15 gauge 2 inch) in such a way that the effects of venous stasis can be avoided. Proof of this statement lies in the material which follows.

Five collecting vials, each containing the same kind and amount of anticoagulant, were filled to the 10 c.c. mark from the 15 gauge 2 inch needle inserted in the jugular vein. Venous pressure was continuous throughout the fillings and was accomplished by thumb pressure 5 to 6 inches below the needle and in degree was sufficient only to allow blood to run from the needle. Samples were allowed to settle in the vials at room temperature and then were shaken mechanically in a uniform manner. A Wintrobe tube was filled from each vial and placed in a rack to insure its perpendicularity. Results were:

Aliquot No.	M_5‡	t_{M_5}	$\angle^{8}M_5$
1	26 mm.	15-20 min.	30° 30′
2	20 mm.	10-15 / 15-20 min.	37° 20′
3	18 mm.	10-15 min.	40° 20′
4	19 mm.	15-20 min.	39° 40′
5	24 mm.	15-20 min.	32° 20′

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‡*Definitions:*

M_5 = Maximum five-minute descent in millimeters of the line of demarcation between cells and plasma.

t_{M_5} = Times in minutes between which M_5 occurs. Since with the photographic apparatus one minute is the equivalent of 3 mm., this time-distance ratio is retained for the "field" technique.

$\angle_{M_5}$ = Angle opposite the horizontal side of a right triangle having M_5 as the perpendicular side and five minutes, or 15 mm., as the horizontal side.

Aliquot No.	M_5	t_{M_5}	$\angle^s{}_{M_5}$
1	19 mm.	10-15 min.	39° 40′
2	21 mm.	15-20 min.	36° 00′
3	15 mm.	20-25 min.	45° 00′
4	16 mm.	10-15 min.	44° 30′
5	17 mm.	15-20 min.	41° 20′

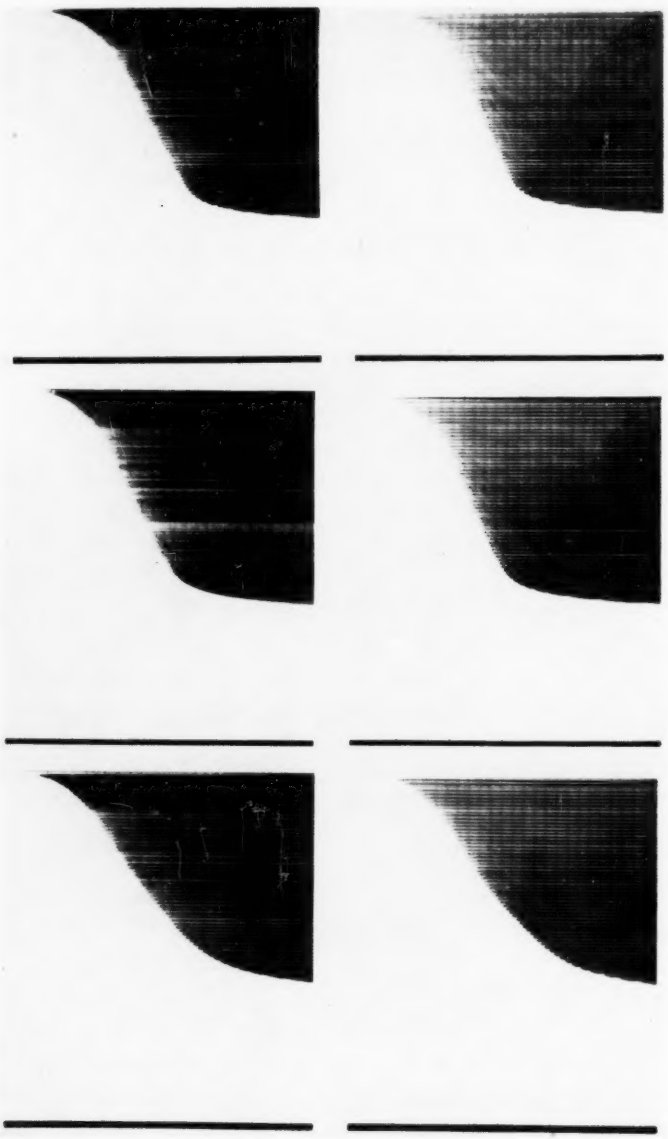

Fig. 1.

To show what happens when the thumb is removed after filling each collecting vial and is reapplied before filling the next:

Aliquot No.	M_5	t_{M_5}	$\angle^s{}_{M_5}$
1	22 mm.	15-20 min.	34° 40′
2	23 mm.	15-20 min.	33° 30′
3	26 mm.	10-15 min.	30° 20′
4	22 mm.	10-15 min.	34° 40′
5	23 mm.	10-15 min.	33° 30′

Another example:

Aliquot No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%*
1	18 mm.	20-25 min.	40° 10′	39
2	18 mm.	15-20 min.	40° 10′	38.3
3	15 mm.	20-25 min.	45°	39½
4	18 mm.	20-25 min.	40° 10′	39½
5	18 mm.	15-20 min.	40° 10′	38½

This method is an improvement over continuous application of the thumb, but it still does not yield as comparable results as the technique with no stasis. In other words, if no thumb pressure is applied, except just enough to raise the vein to insert the needle, and if, after a moment's hesitation to allow free flow through the vein, a single sample is drawn into a clean dry glass syringe, and if five 10 c.c. aliquots are immediately supplied from the syringe, then results of determinations of M_5, t_{M_5}, and $\angle^s_{M_5}$ as also photographed curves of sedimentation from these aliquots will be comparable, for example:

Aliquot No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	24 mm.	15-20 min.	32° 30′	34.5
2	23 mm.	15-20 min.	33° 30′	34.5
3	24 mm.	15-20 min.	32° 30′	35.0
4	25 mm.	15-20 min.	31° 20′	34.0
5	25 mm.	15-20 min.	31° 20′	34.5

This evidence, which may be repeated at will, is convincing enough to me that stasis of the vein at the time of withdrawal of a blood sample for the measurement of sedimentation should be avoided.

Delay.—Next let us consider the effects of delay in starting measurement following bleeding. Fig. 1, derived from three-paired samples, taken from one bleeding is typical of results. The oxalate mixture of Heller and Paul[4] was the anticoagulant used.

Ex. No.	Time after bleeding	em†	t_{em}	$\angle^s_g$
1	60 min.	28 mm.	12.8 min.	23° 00′
2	60 min.	28 mm.	11.8 min.	22° 30′
3	114 min.	30 mm.	11.8 min.	24° 40′
4	114 min.	29 mm.	11.0 min.	24° 10′
5	139 min.	20 mm.	10.3 min.	31° 30′
6	139 min.	21 min.	11.0 min.	31° 10′

Ex. No.	M_5	t_{M_5}	$\angle^s_{M_5}$	V%
1	28 mm.	10-15 min.	28° 30′	31
2	29 mm.	10-15 min.	27° 40′	32
3	28 mm.	10-15 min.	28° 30′	30.7
4	29 mm.	10-15 min.	27° 40′	31
5	19 mm.	10-15 min.	38° 50′	31
6	21 mm.	10-15 min.	36° 00′	30.7

*V% = Volume per cent of packed cells.

†em = The perpendicular distance from the estimated point of change between the vertical flexion and horizontal flexion of the photographically recorded sigmoid curve of sedimentation of a sample and the top line of the photograph representing 100 mm. of blood column height, i.e., estimated "fall" in millimeters at the point of change.

t_{em} = Time in minutes between the time of filling of the Wintrobe tube and the time that the point of change between the vertical flexion and horizontal flexion of the photographically recorded sigmoid curve of sedimentation of a sample occurs (one minute = 3 mm.).

$\angle_g$ = The least angle between the steepest slope of the sigmoid curve of sedimentation of a sample and perpendicularity.

The following is an example where potassium oxalate alone is used as an anticoagulant (Fig. 2). Approximately a ninety-minute interval exists between Example 1 and Example 2, both of which are from the same sample of blood.

Ex. No.	em	t_{em}	$\angle^{s}_{g}$
1	27 mm.	11.0 min.	26° 50′
2	8 mm.	9.5 min.	69° 20′

Ex. No.	M_5	t_{M_5}	$\angle^{s}M_5$	V%
1	29 mm.	5-10 min.	27° 40′	40.7
2	8 mm.	5-10 min.	71° 40′	39.7

There appears to be a slowing of the rate of separation as time progresses. Examination of many samples suggests that with the ammonium and potassium oxalate mixture of Heller and Paul there may be a slight increase in rate preceding the slowing effect. However, tests run within approximately two hours yield fairly comparable results with this anticoagulant mixture. With some anticoagulants, particularly potassium oxalate, the effects of delay are apparently more pronounced and seemingly the more anticoagulant used the sooner slowing takes place.

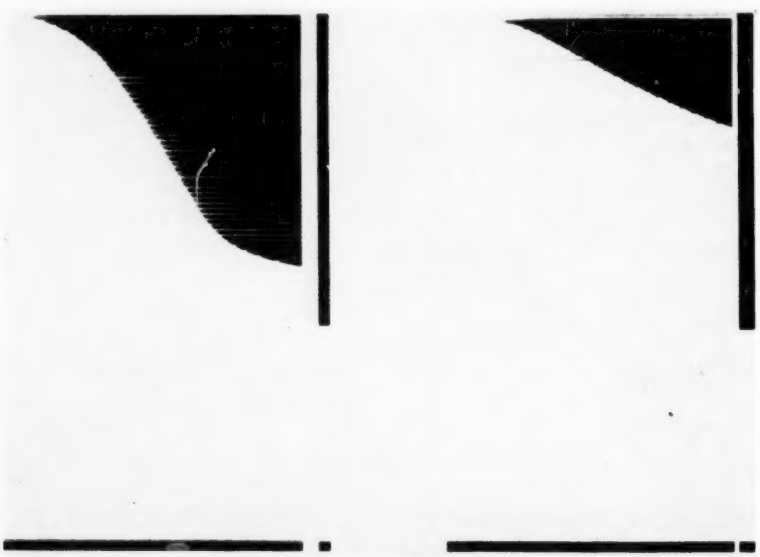

Fig. 2.

Temperature.—Multiple determinations of rates at almost any single temperature are repeatable, but in the interest of standardization one should obviously not attempt comparison of samples at widely different temperatures. For example:

Aliquot No.	Temperature	M_5	t_{M_5}	$\angle^{s}M_5$	V%
1	38° C.	29 mm.	10-15 min.	27° 30′	36.5
2	38° C.	30 mm.	10-15 min.	27° 00′	36.5
3	26° C.	22 mm.	10-15 min.	34° 40′	37
4	26° C.	22 mm.	15-20 min.	34° 40′	36.7
5	6° C.	3 mm.	85-100 min.	78° 20′	37 +
6	6° C.	2.5 mm.	75-80 min.	80° 10′	37.3

With the temperatures tested the sedimentation rate increases as temperature rises. As yet no evidence has appeared to convince me that body temperature has any advantage over room temperature, or vice versa. No attempt has been made to demonstrate any mathematical relationship between temperature and sedimentation, although the possibility of such may exist.

Containers.—The reproducibility of the following tubes was tested:

Blood column—100 × 3 mm.:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$	V%
1	18 mm.	20-25 min.	40° 20′	39.3
2	18 mm.	15-20 20-25 min.	40° 20′	39.3
3	18 mm.	15-20 min.	40° 20′	39.5
4	17 mm.	15-20 min.	42° 00′	39
5	18 mm.	15-20 min.	40° 20′	39

Blood column—200 × 3 mm.:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$
1	29 mm.	15-20 min.	27° 40′
2	30 mm.	15-20 min.	27° 00′
3	28 mm.	10-15 min.	28° 30′
4	28 mm.	10-15 min.	28° 30′
5	27 mm.	10-15 15-20 min.	29° 20′

Blood column—100 × 6 mm.:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$
1	16 mm.	20-25 min.	43° 40′
2	16 mm.	25-30 min.	43° 40′
3	17 mm.	25-30 min.	42° 00′
4	16 mm.	20-25 min.	43° 40′
5	16 mm.	20-25 min.	43° 40′

Blood column—200 × 2 mm.:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$
1	6 mm.	30-35 min.	67° 10′
2	18 mm.	25-30 30-35 min.	40° 20′
3	19 mm.	20-25 25-30 min. 30-35	38° 50′
4	19 mm.	25-30 min.	38° 50′
5	14 mm.	30-35 min.	46° 10′

Another example of 200 × 2 mm.:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$
1	23 mm.	10-15 15-20 min.	33° 40′
2	15.5 mm.	40-45 min.	44° 30′
3	24 mm.	20-25 min.	32° 30′
4	23 mm.	20-25 min.	33° 40′
5	21 mm.	20-25 min.	36° 00′

As can be seen all have fair reproducibility except the 200 × 2 mm. columns. Even with many efforts to fill uniformly these tubes, erratic results ensued. Despite these efforts I still feel that it is more reasonable to believe that variations are in a large part the result of discrepancies in tube filling and not of capillarity in a 2 mm. tube. Horse blood will not flow from an

inverted 3 mm. tube and seldom from a 6 mm. tube, yet with these bores replication is possible. Why the difference? Of course, with the 200 mm. tubes longer needles had to be employed for filling. Since these needles are No. 16 gauge and their outer diameter is 1+ mm., they nearly occlude the 2 mm. bore. In the necessary manipulation of the filling of the tube with a needle of this size, in all probability disturbance to the blood is greater in some fillings than in others, resulting in more complete breaking up of clumps of cells. Since the same needles can be used for filling larger bore tubes and replicate results ensue, it is thought that they are not in fault. Smaller bore needles, though they too offer disadvantages from an agitation standpoint, will have to be tried.

Next a comparison of 100 × 3 mm. and 200 × 3 mm. columns was made on aliquots of the same sample of blood. All tubes were filled with No. 16 gauge 11 inch needles:

Blood column—100 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
1	16 mm.	20-25 min.	43° 40′
2	15 mm.	20-25 min.	45° 00′
3	17 mm.	25-30 min.	42° 00′

Blood column—200 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
4	20 mm.	20-25 / 25-30 min.	37° 30′
5	22 mm.	20-25 min.	34° 50′
6	21 mm.	20-25 min.	36° 10′

Four 200 × 3 mm. tubes were filled to different levels:

Aliquot No.	Column height	M_5	$^{t}M_5$	$\angle^{s}M_5$	$\%M_5$*	$\angle^{s}\%M_5$
1	200 mm.	22.5 mm.	20-25 min.	34° 00′	11.25 mm.	52° 20′
2	150 mm.	18.5 mm.	10-15 min.	39° 30′	12.3 mm.	49° 50′
3	100 mm.	24 mm.	10-15 min.	32° 30′	24 mm.	32° 30′
4	50 mm.	13 mm.	5-10 min.	48° 30′	26 mm.	30° 20′

As can be seen by examination of these trends the actual maximum five-minute drop in the line of demarcation between cells and plasma is greater in 200 mm. columns than in 100 mm. Results in tubes of like bore filled to four different levels suggest that M_5 may be less and less as the height of the column is decreased.

If, however, one were to reduce the actually obtained values of M_5 to a percentage basis, then values of $\%M_5$ are not equal but increase as the column height decreases—additional indication that there is not a simple arithmetical relationship between column height and maximum drop. Attempts to demonstrate some other type of mathematical relationship will have to be left for future work—such a relationship theoretically might exist.

For an example of comparison of like columns of different diameter:

Blood column—100 × 3 mm.:

Aliquot No.	M_5	$^{t}M_5$	$\angle^{s}M_5$
1	15 mm.	25-30 min.	45° 00′
2	15 mm.	20-25 min.	45° 00′
3	17 mm.	20-25 min.	42° 00′

*$\%M_5$ equals M_5 reduced to a percentage basis.

Blood column—100 × 6 mm.:

4	16.5 mm.	20-25 min.	42° 50′
5	16.5 mm.	20-25 min.	42° 50′
6	17 mm.	20-25 min.	42° 00′

Results are close.

As examples of the effects of deviations from perpendicularity:

Aliquot No.	Angles	M_5	$^{t}M_5$	$\angle^{s}M_5$	V%
1	90°	24 mm.	10-15 min.	32° 30′	34.7
2	80°	31 mm.	5-10 min.	26°	34.3
1	90°	21 mm.	15-20 min.	36° 10′	38.00′
2	80°	28 mm.	10-15 min.	28° 30′	38.00′

Five-minute readings for the angulated tubes were read directly from the graduations of the tubes. Little calculation is necessary to show that despite this, values of M_5 are greater than those of the corresponding perpendicular tubes.

To summarize, longer columns yield greater actual values of M_5. M_5 is not directly proportional to column height, for when values of M_5 are reduced to percentage drops, these are greater in shorter columns. There is apparently no significant difference between columns of 3 mm. diameter and columns of 6 mm. diameter. Two millimeter columns should be avoided. Small deviations from perpendicularity are apparently sources of discrepancy.

Shaking.—As one may have noticed in the review of literature on agitation prior to starting measurement of sedimentation,[1] too little stress has been placed upon the necessity of uniform dispersion of elements before measurement is begun. It was first reasoned that, if multiple aliquots from the same sample of blood were shaken to a point where they would yield fairly close values of volume per cent, their sedimentation rates should agree. The fallacy of such reasoning was soon clearly demonstrated. Such reasoning would apply to a simple sedimenting system where the dispersed elements remain discrete, but not to blood in which the phenomena of rouleaux formation and agglutination take place, and more especially not to blood in which this aggregating tendency is very pronounced, as is the case with horse blood. It has been reported that no aggregation occurs intravascularly in healthy human beings but such has not been reported in healthy horses. In fact, Fahraeus[5] states that close examination of the finer vessels of the mesentery of the horse reveals that the corpuscles are so strongly aggregated that they do not separate during flowing. No suspension exists. The erythrocytes flow as a compact stream in the middle of the vessel, and only when the stream is slowed by pressure on the supplying arteries does this string show granulated outlines and finally break to pieces. Whether Fahraeus' observation can be classed as evidence that a certain degree of aggregation occurs intravascularly in the healthy horse is certainly debatable. The very removal of mesentery from the abdominal cavity for examination may have in itself caused a certain degree of slowing in the vessels, this favoring rouleaux formation. More especially might this be true since the aggregating tendency is naturally very great in horse blood.

The problem of obtaining a uniform suspension of elements, whether singular, rouleaux, or aggregates of rouleaux, has been indeed a difficult one. Naturally, the first method attempted consisted of filling tubes as soon as possible

after bleeding, the aliquots being agitated by hand until the samples for filling the Wintrobe tubes were drawn. Such yielded erratic results.

The next method tried consisted of allowing the collecting vials to set until separation was fairly complete, usually ten to twenty-five minutes, and then shaking them as uniformly as possible by hand. This method was not dependable.

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$	V%
1	21 mm.	15-20 min.	36° 00′	35–
2	24 mm.	15-20 min.	32° 30′	35+
3	23.5 mm.	15-20 min.	33° 00′	35

Striking the collecting vial upon a rigid surface only created more incomparable results:

Aliquot No.	M_5	t_{M_5}	$\angle^s M_5$	V%
1	20.5 mm.	10-15 min.	36° 40′	35
2	21 mm.	5-10 min.	36° 00′	35+
3	15 mm.	10-15 min.	45° 00′	35

In face of erratic results with manual agitation, mechanical means were attempted. Several styles of mechanical agitators were constructed and tried. Finally, one was built[2] with a vial holder suspended from four 7¾ inch spring steel arms. This was driven in such a way as to yield a ¾ inch path of motion over an arc of 7¾ inch radius, yet, since four arms were used, the vial remained in a perpendicular position. The fact that the ends of the stroke were slightly higher than the center of the arc is thought to be an added assistance in overcoming the effects of the force of gravity (which theoretically is ever present and must be counteracted before dispersion can take place). When the path of the arc was more shallow (12 inch radius), layering effects were frequently encountered. At present the speed is 564 strokes per minute. It is felt that since fairly comparable results can usually be obtained when tubes are filled from different heights of replicate aliquots of the same sample of blood, in all probability this agitator is at least practically sufficient.

Aliquot No.	Height	M_5	t_{M_5}	$\angle^s M_5$	V%
1	¾	19 mm.	20-25 min.	38° 50′	41.7
2	½	18 mm.	20-25 min.	40° 20′	42
3	¼	17 mm.	20-25 min.	42° 00′	41.7
4	0	19 mm.	20-25 min.	38° 50′	42

Additional evidence of its efficiency is noted when five Wintrobe tubes are filled from the bottom of the same 10 c.c. sample:

Tube No.	Height	M_5	t_{M_5}	$\angle^s M_5$	V%
1	0	16 mm.	20-25 min.	43° 40′	38
2	0	16 mm.	20-25 min.	43° 40′	38
3	0	16 mm.	15-20 min.	43° 40′	38.3
4	0	17 mm.	15-20 min.	42° 00′	38+
5	0	16 mm.	15-20 min.	43° 40′	38+

The foregoing examples were from samples which were allowed to settle; each sample was shaken for fifteen minutes by placing the collecting vials into the shaker one minute apart and filling the tubes one minute apart.

The first attempts at arriving at a safe time of mechanical agitation for samples immediately placed in the shaker yielded results shown in Fig. 3. Data from these examples:

Ex. No.	Time shook	em	t_{em}	$\angle^{s}_{g}$
1	2 min.	31 mm.	10.5 min.	22° 10′
2	3 min.	29 mm.	9.3 min.	20° 00′
3	3.5 min.	29 mm.	8.3 min.	17° 50′
4	4.5 min.	26 mm.	7 min.	21° 30′
5	5.5 min.	23 mm.	7 min.	17° 30′

Ex. No.	M_5	t_{M_5}	$\angle^{s}M_5$	V%
1	27 mm.	5-10 min.	29° 20′	31.8
2	29 mm.	5-10 min.	27° 20′	31.8
3	33 mm.	5-10 min.	24° 40′	31
4	35 mm.	5-10 min.	23° 20′	31
5	39 mm.	5-10 min.	21° 10′	31.3

As can be seen, five and one-half minutes at a speed of about 600 strokes per minute was apparently not sufficient to reduce aggregated elements to at least minimum size.

To determine whether results might be comparable if samples were drawn, immediately placed in the shaker, and agitated for fifteen minutes:

Aliquot No.	M_5	t_{M_5}	$\angle^{s}M_5$	V%
1	17 mm.	25-30 min.	42° 00′	44
2	16 mm.	20-25 min.	43° 40′	43.5
3	13 mm.	20-25 min.	48° 20′	44
4	20 mm.	25-30 min.	37° 20′	44
5	20 mm.	25-30 min.	37° 20′	43.5

Results were still too variable.

If samples were immediately placed in the shaker and agitated for forty minutes:

Aliquot No.	M_5	t_{M_5}	$\angle^{s}M_5$	V%
1	23.5 mm.	10-15 min.	33° 00′	37–
2	23.5 mm.	10-15 min.	33° 00′	36.3
3	22 mm.	15-20 min.	34° 40′	36.7
4	23.5 mm.	10-15 min.	33° 00′	36.5
5	23.5 mm.	10-15 min.	33° 00′	36+

Results agree very well.

Apparently a certain amount of aggregation takes place even during shaking, and apparently a certain amount of time following bleeding must pass before agreeable replicate values can be obtained. From a practical standpoint, if equally as good results can be obtained by allowing the blood to separate before agitation as by prolonged agitation, the logical choice is to allow settling to take place first. There is additional experience in favor of this choice. In samples of very slow blood, agitation, if commenced immediately after bleeding, must be continued longer than in samples from fast blood. In slow blood one is always reasonably doubtful about reaching that phase of agitation where replication is possible. Where slow blood is allowed to settle first, in no case so far has there been evidence to suggest that agitation for fifteen minutes was insufficient.

It was first thought that if blood was allowed to separate first, speed alone might bring about uniformity of suspension. Blood was allowed to settle and

then was placed in the agitator between 500 and 600 strokes per minute for varying times. There was no visible hemolysis at this speed while at faster speeds there was occasional hemolysis in some pathologic cases.

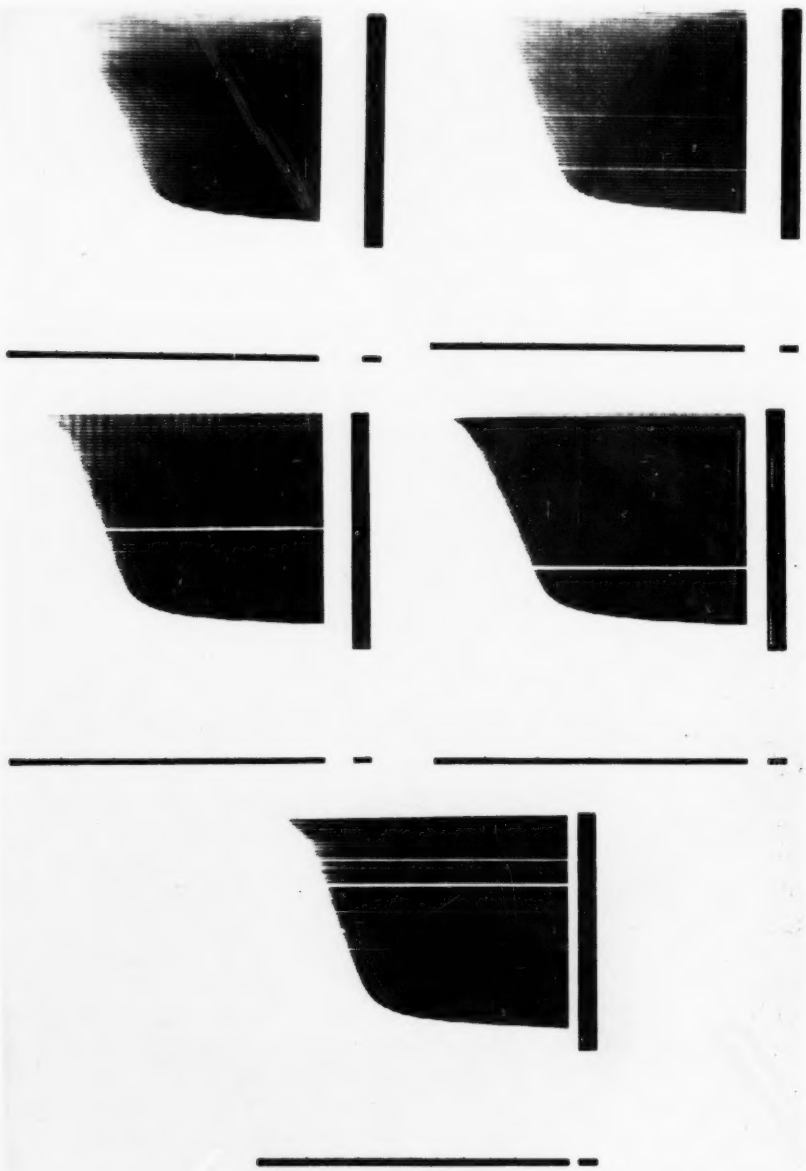

Fig. 3.

Results were similar to:

Aliquot No.	Time Shaken	M_5	$^{t}M_5$	$\angle^{s}M_5$	V%
1	1 min.	12 mm.	15-20 min.	50° 30′	42½
2	5 min.	16 mm.	30-35 min.	43° 40′	43–
3	10 min.	18 mm.	20-25 min.	42° 40′	42–
4	15 min.	19 mm.	20-25 min.	38° 40′	42½

Shaking for thirty minutes after the samples have been allowed to separate yields no more comparable figures than shaking for fifteen minutes. In more rapid samples shaking for five minutes will yield comparable values, but I have greater confidence in fifteen minutes as a routine shaking time.

It was earlier mentioned[2] that variations in t_{em} and the limits of t_{M_5} were encountered even when values of em and M_5 were not far off. Volume percentages were within narrow limits, t_o-t_o* was carefully established, and all calculations and measurements were checked. At first it was thought that the mechanical agitation was at fault. Since at no time has it been possible to demonstrate microscopically that settled blood can be separated by agitation to a state of single cells—because rouleaux formation is so rapid in the equine species that shed blood no matter how quickly examined after bleeding cannot be found without agglutination—no definite proof of the efficacy of any type of agitation has been possible as yet. Means other than the microscope will have to be used.

However, since with some methods of filling the measuring tubes very close results were obtained while with others results were not so close, it was reasoned that in all probability the mechanical agitation merely reduced the solid elements to a certain degree of dispersion but not to the discrete state and that methods of filling measuring tubes were at fault. The early practice of removing the collecting vial from the shaker before withdrawal of blood for filling the measuring tube was stopped, and in its place was substituted the practice of withdrawing blood from the vial while the shaker was in motion, this being possible because of the short stroke of the apparatus. In addition to this, syringes attached to blunt No. 18 gauge 4½ inch needles were used in place of the earlier used glass pipettes furnished with the Wintrobe tubes, the latter being very subject to breakage when inserted into the moving vial. Results were no better.

Next it was reasoned that the suction of drawing the blood into a syringe (used earlier in the course of this problem) and the pressure necessary for its expulsion into a tube were probably in reality means of agitation in themselves; and if no uniformity existed in this practice, they might be responsible for some of the discrepancies observed. It was further reasoned that if this source of possible variation could be minimized, closer results would ensue. Since the hubs of these long-filling needles were round and fairly large, the rubber bulbs which are furnished with the Wintrobe filling pipettes were attached and tried. Before trial these bulbs were selected as to volume and degree of firmness, and only those which were comparable were employed. By using this equipment and placing more stress on forming a uniform "habit" of filling tubes, closer results of em, t_{em}, M_5 and t_{M_5} were obtained.

The problem of t_{M_5} has, however, not yet been completely solved, but a little reasoning reveals that since limits of t_{M_5} never pass beyond the next five-minute brackets and since, even if the limits of t_{M_5} are within different brackets, in practice em, t_{em}, $\angle^8_g$, M_5 and $\angle^8_{M_5}$ may be very close, this discrepancy is not too serious. After all t_{M_5} merely is a wide range within which t_{em} lies. Usually

*Time of filling the tube.

if t_{em} is close to one or the other of the limits of t_{M_5} in results where there is variation in t_{M_5}, it will be in the direction of t_{em}. Also where two readings of M_5 are close, t_{em} usually lies nearer the middle of these five-minute limits. In cases where M_5 lies between both five-minute limits, as it frequently does, t_{em} in most cases lies closer to the center five minute time. Since in practice t_{em} is usually not affected more than one minute, and rarely two minutes or more, and since in all probability $\angle^{s}_{g}$ in the photographic technique and $\angle^{s}_{M_5}$ in the "field" technique are better estimates of the speed of settling than values of either I_{em}* or I_{M_5}†, this deviation in t_{M_5} is thought not serious. Also since, if t_{em} happens to be close to one or the other of the limits of t_{M_5}, small variations in t_{em} may be sufficient to change the limits of t_{M_5}, in replicate sampling variations in values of t_{M_5}, apparently due to discrepancies in filling the measuring tubes, are not considered of distracting value.

Since replicate results agree very closely with the previously described technique of agitation and tube filling, this method is thought to be fairly dependable.

Volume Per Cent and Sedimentation.—Now comes a phase of this work which is indeed interesting and which I feel cannot be answered in a thesis purely on technique, but it might be in a problem of "apparently normal ranges." What is the relationship between the quantity of formed material and the rate at which this material separates out? Since it is quite universally recognized that the cells do not settle discretely, what effect does the quantity of formed elements have upon rouleaux formation and agglutination?

Rouleaux formation has been said to be merely a type of agglutination in which the cells, because of their specific shape, take on a nummular appearance, other physical factors apparently being similar. Is such unreasonable? Rouleaux, as observed microscopically, are said to attain a certain average length and from thence on agglutination is of rouleaux and not single cells. It has even been suggested[5] that agglutination continues until the whole mass of cells seems to form a spongelike architecture.

It has also been suggested by Balachowsky[6] that:

1. Red blood cells forming rouleaux and conglomerates constitute a network which is the skeleton of the blood.

2. Under the influences of weight and through a series of local depressions there occurs a general heaping of the blood skeleton on the bottom of the receptacle.

3. This blood skeleton is not deprived of solidarity for (a) the configuration of a given portion may remain unchanged for a long period of time. (b) The free upper border of the bulk of the erythrocytes preserves its shape for a long period. (c) There exists a force of resistance against the collapse of the blood skeleton other than viscosity.

4. The solidarity of the blood skeleton, being a function of the tonus of its constituents, is a new factor denominated "hematonia."

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*I_{em} = An index $\frac{em}{t_{em} \times 3}$.

†I_{M5} = An index calculated by dividing the "falls" in millimeters between which M_5 occurs by their corresponding times × 3 (3 mm. = one minute).

Balachowsky feels that sedimentation of erythrocytes is not, as has been assumed, merely a simple phenomenon of sedimentation, but a more complicated reaction in which the effects of heaping play an important part. For this reason, he would dominate the phenomenon as the sedicompilation of erythrocytes.

If one closely watches a Wintrobe tube of horse blood, he can observe that the edges of the column of blood present a certain, ragged appearance, sometimes even before much settling has taken place. Is it unreasonable to assume that this is part of the agglutination phenomenon and that this effect may be general throughout the column? Another common place that this may be observed is in the pipette of a Dare hemoglobinometer. The layer of blood is sufficiently thin for one to watch the gradual agglomeration and settling.

Of course, one can observe a certain limitation to the size of rouleaux and clumps under a cover slip. The physical boundary of the blood column in one direction (height) is a matter of microns, and this in itself physically limits, to some extent at least, the degree of agglomeration. The dilution necessary to demonstrate rouleaux probably is in itself a limiting factor. However, cover-slip specimens of a sample of blood may show a certain limit to agglomerate size while tubes of the same sample may present evidence of mass agglomeration. In other words, in samples examined under a cover slip, mass agglomeration is quite free in two dimensions, but somewhat limited in the third, while in tubes, agglomeration is practically unlimited in three dimensions, at least before mass collapse.

Should it be said that the descent of the line of demarcation between cells and plasma represents a measure of size of agglutinates, or should it be said that the fall of this line of demarcation represents a measure of mass agglutination and mass collapse?

As to the effect of the quantity of formed material, cannot one say that, being one of the factors controlling the degree of agglutination as observed microscopically, it probably is one of the controlling factors of the degree of mass agglutination? Examinations of cover-slip preparations of various dilutions of cells and maximum five-minute falls on the same sample seem to substantiate this, as for example:

	V%	M_5	t_{M_5}	$\angle^s M_5$
Original sample:	43	21.9 mm.	10-15 min.	34° 40′
Volume altered to:				
"	48	14 mm.	10-15 min.	46° 10′
"	39	25 mm.	5-10 min.	31° 30′
"	29.5	48 mm.	0-5 min.	17° 30′
"	20	61 mm.	0-5 min.	13° 50′
"	10.5	72 mm.	0-5 min.	11° 50′

Since the degree of agglutination was too great to count rouleaux and estimate the number of cells per rouleaux, the blood of the same animal was diluted (with 0.9 per cent sodium chloride) so that such might be accomplished. As expected, the size and number of agglomerates diminished with dilution, next the size and number of rouleaux diminished, until finally all single cells existed. Since the border of cells and plasma became more and more hazy the greater the dilution, actual estimations of M_5 were given up.

In dilutions showing hazy plasma, it will be noticed that that portion of the elements which does settle out rapidly gradually diminishes in volume as dilution increases, until finally the suspension comes apparently to its maximum stability. This "titer" corresponds roughly at least to the point at which only single cells are observed in the cover-slip preparations.

As purely a general observation there seems to be a concentration of elements in apparently healthy blood where the point of change in the sigmoid curve roughly numerically equals the percentage volume of elements, namely, at about 33⅓ per cent or ⅓, of the column height. The point of change in "normal" samples is seemingly less than 33⅓ mm. when the volume per cent is greater than 33⅓, and vice versa. Proof of this observation has not been attempted in this problem nor has any attempt been made to relate mathematically the point of change of curves of sedimentation with volume percentages because it is thought that such must entail examination of many "normal" samples and their adjustment to various concentrations—a task which would in itself entail more time and effort than can be afforded this thesis and could well be included in a study of normals. Nevertheless, Fahraeus[5] must have been thinking of a somewhat similar observation when he made this statement: "In general the sinking velocity of the upper corpuscle layer is diminished when it has sunk ⅓rd of the height of the blood column, i.e., in the tubes used by me (150 mms.) when the line of demarcation has descended 50 mms."

To theorize, if a mathematical relationship can be proved to exist between the point of change of the sigmoid curve representing the descent of the line of demarcation between cells and plasma and the percentage volume of formed elements, then with this knowledge, if the point of change is not as expected for a particular percentage, its deviation from the expected might reflect alterations produced by factors other than the volume percentage of elements. Such alterations might be estimated without standardization of the quantity of formed elements for the particular sample. Such no doubt would be an advantage.

To summarize in a general way, we seem to have least settling at dilutions where cells are discrete; a gradual increase in settling of at least a portion of the cells (probably as rouleaux or small clumps) as dilution becomes less; a point where maximum speed of separation seems to occur; then a gradual decrease in separation speed (as determined by the descent of the line of demarcation between cells and plasma) as concentration increases, there possibly being a mathematical relation between the slowing of the speed of separation and the increase in concentration.

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BLOOD CLOT RETRACTION*

I. Measurement of the Extracorpuscular Volume of the Clot

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DIRECT observation of the blood clot may reveal gross evidences of friability and deficient retraction. However, quantitative measurements are required to compare retraction of the blood clot of different patients, or of that of the same patient over a period of time. A method for estimating the degree of clot retraction, called the "serum volume index," was introduced by Boyce and McFetridge.[1] A quantity of blood, preferably 3 c.c., was collected in a graduated centrifuge tube and allowed to stand at room temperature. At the end of four hours the total volume of the specimen was noted, the clot was removed, and the residual volume of serum was recorded. In normal subjects the volume of serum was stated to be equivalent to 50 per cent of the volume of the specimen. There was a marked diminution of the serum volume in some jaundiced patients who suffered from abnormal bleeding. The authors expressed the deficiency of clot retraction in terms of the "serum volume index," which was obtained by dividing the volume of the serum by one-half of the total volume of the blood specimen. The index for normal subjects was 1.00, and values less than one were considered as indicative of a tendency to bleed. It was observed that if anemia were present, the serum volume would normally be greater than half the blood volume, and a correction based on the erythrocyte count was made in calculating the index.[2] It appears to us that in addition the mean volume of the red blood corpuscles and increases in the number of leucocytes may influence the results of the test. Nevertheless, the serum volume index is a valuable bedside test for the detection of deficient clot retraction.

Macfarlane[3] introduced a new method for measuring clot retraction which makes use of a graduated centrifuge tube into which is fitted a cork bored to receive a glass rod. The glass rod measures about 0.5 inch shorter than the tube and has a small expansion about 0.5 inch from the lower end. To perform the test, 5 c.c. of blood obtained by venipuncture are immediately introduced into the tube, the glass rod and cork are fitted, and the tube is placed in a water bath at 37° C. One hour after a firm clot has formed, the tube is removed from the bath, the clot is carefully freed and removed on the glass rod. The volume of serum is measured directly, and retraction is expressed in terms

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of the percentage of the serum volume. The author found that the normal serum volume per cent fluctuated between 43.9 and 65.5 per cent. No correction was made for anemia.

The "clot retraction index" introduced by Mettier and Witts in 1939[9] is performed in a manner similar to that described by Macfarlane, except that a correction for anemia is made. The packed cell volume is added to the serum volume per cent and the sum is divided by two. In normal subjects the "clot retraction index" varies from 38 to 60. We have found the "clot retraction index" to be a useful clinical test.[6, 7]

In the course of a study of the various factors influencing the volume of the clot, we have found it convenient to express the character of the clot in terms of the extracorpuscular volume of the clot, which may be defined as the difference between the volume of the clot and the volume of the cells contained therein. It is our purpose to record the results of observations made in normal subjects and in patients suffering from abnormal bleeding.

METHOD

Slight modifications of Macfarlane's method for examining the clot were made. The upper end of a copper wire about 1 mm. in diameter is attached to a cork. The free end of the wire is bent in the shape of a hook. An arbitrary amount of venous blood, usually 5 c.c., is carefully transferred to a graduated centrifuge tube, and the cork and wire are fitted so that the hook is immersed in the upper layers of the specimen. After coagulation of the blood has occurred, the tube is placed in a water bath (37° C.) for one hour. It is then taken from the bath, the total volume of the specimen is recorded, the clot is elevated, allowed to drain and then removed. The residual volume of serum is measured, and when it is subtracted from the total volume of the specimen, the clot volume remains. To obtain the volume per cent of the blood clot, the observed volume of the clot is divided by the total volume of the specimen, and the quotient is multiplied by 100. (The measurement of the packed cell volume, including all cellular elements, is obtained by centrifugalization of a separate specimen of oxalated blood at 3,000 r.p.m. for thirty minutes.) The difference between the packed cell volume in per cent and the clot volume in per cent is the per cent of the total blood specimen occupied by the extracorpuscular volume of the clot. A value of zero implies that the clot is composed entirely of cells, and hence that excellent retraction has occurred. A high figure indicates that a large amount of extracorpuscular fluid is retained in the clot and that retraction is deficient. Occasionally, the clot volume is slightly smaller than the packed cell volume. This apparent discrepancy could be due to the inherent errors of the method, or it may be that fluid has been expressed from the cells, or that the spatial relationships of the cells are different when they are packed by centrifugal force in contrast to the agglomerations brought about by the natural retraction of the blood clot. Whatever the cause of this phenomenon, it always indicates excellent clot retraction.

Errors of the Method.—The inherent error of the method when tested by serial examination is approximately 5 per cent. The presence of the wire within the clot introduces a constant error of less than 0.5 per cent, which for con-

venience is neglected in the calculations. Occasionally, some of the cells may escape from the clot and settle at the bottom of the tube. This is frequently seen in specimens obtained from patients suffering from thrombocytopenia, polycythemia vera, and congenital hemolytic icterus. In these instances the serum is centrifugalized and the volume of the clot is corrected by adding to it the volume occupied by the escaped cells. The extracorpuscular volume of such clots will be less by an amount equal to the fluid which would be characteristically occluded in such clots had these cells remained within the clot. Such errors are slight and do not alter the trend of the results.

In all experiments the platelet counts were done on capillary blood after the method of Rees and Ecker, and the prothrombin concentration after the method of Quick.

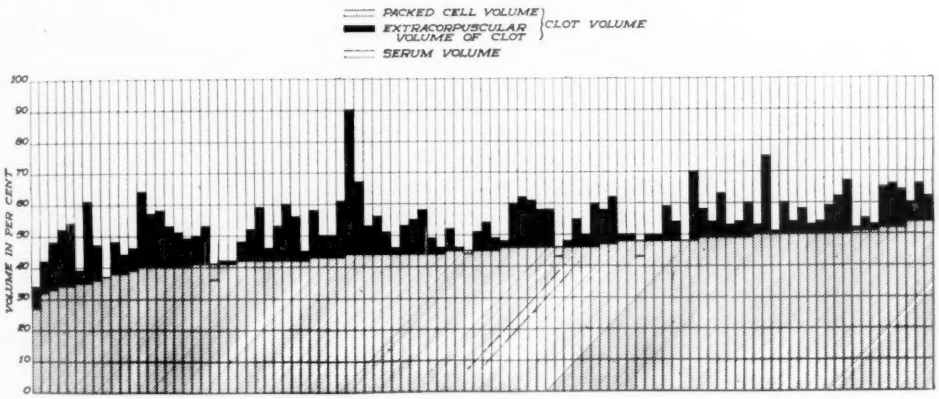

Fig. 1.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 100 normal subjects.

Material of the Study.—Specimens of blood from the following groups of persons were examined:

1. One hundred normal persons in whom the platelet counts were within normal limits (250,000 to 600,000) and in whom the prothrombin concentration was 70 per cent or greater.

2. Forty-two patients suffering from hypoprothrombinemia in whom the platelet counts were greater than 200,000 per cubic millimeter and in whom the prothrombin concentration was 50 per cent or less.

3. Thirty-five patients suffering from thrombocytopenia in whom the platelet count was 200,000 per cubic millimeter or less and in whom the prothrombin concentration was greater than 50 per cent.

RESULTS

The serum volume, clot volume, packed cell volume, and extracorpuscular clot volume expressed in per cent for each of 100 normal subjects are given in Fig. 1. The data for those with hypoprothrombinemia are given in Fig. 2, and for those with thrombocytopenia in Fig. 3. There did not appear to be any significant relationship between the packed cell volume and the extracorpuscular volume of the clot in any of these patients. In Fig. 4 the frequency distribution of the values for the extracorpuscular volume of the clot observed in each

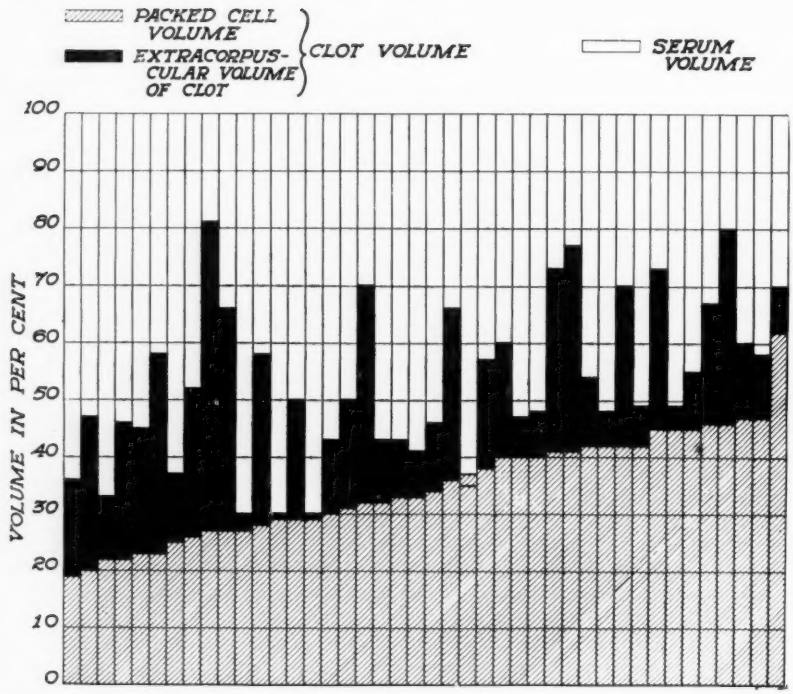

Fig. 2.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 42 subjects with hypoprothrombinemia.

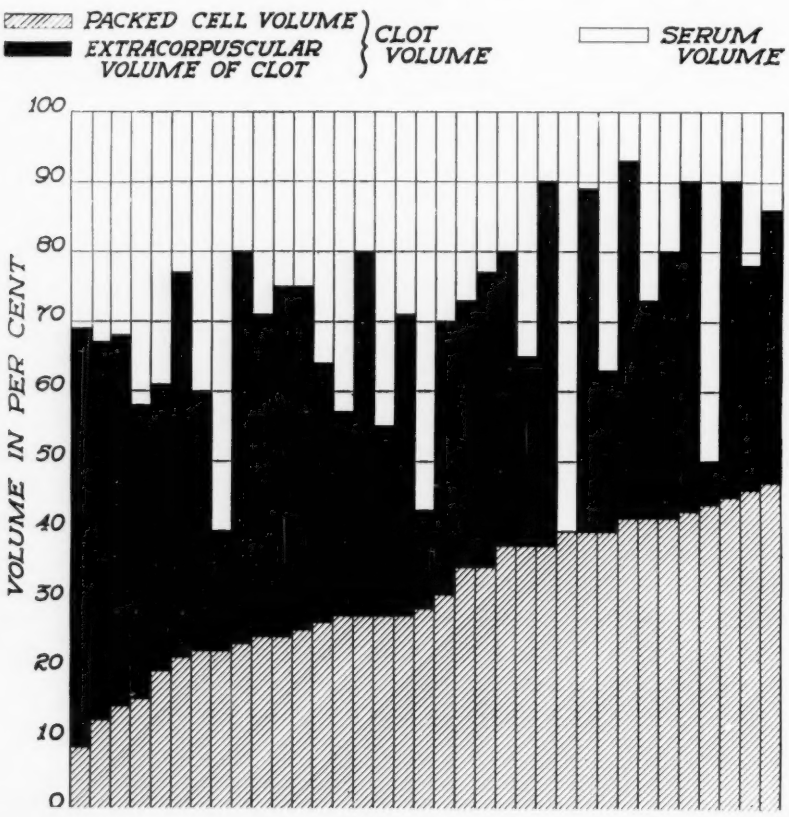

Fig. 3.—Relation between serum volume, packed cell volume, clot volume, and extracorpuscular volume of the clot in 35 subjects with thrombocytopenia.

of the three groups is given. It can readily be seen that the values for the extracorpuscular volume of the clot of many patients with hypoprothrombinemia overlap the normal range, while the values for most of the patients with thrombocytopenia fall outside the normal range. The results of the statistical analysis of these data are given in Table I. The standard statistical method was used in setting up the limits of normality. The limits of significance of the data were arbitrarily set at two standard deviations from the mean, which includes approximately 96 per cent of the observations. The mean is taken at the point of reference. All measures calculated were at least three times their sampling errors. The mean value of the extracorpuscular volume of the clot for the normal group was 9.1 per cent, the range of the values being – 5.9 to 24.1 per cent. The extracorpuscular volume of the clot was greater than 24.1 per cent in 13 of 42 patients with hypoprothrombinemia and in 30 of 35 patients with thrombocytopenia. The respective mean values for these groups were 18.2 and 38.9 per cent.

TABLE I

POINT AND RANGE MEASURES OF THE EXTRACORPUSCULAR VOLUME PER CENT OF THE BLOOD CLOT*

	MEAN	σ_x	M $\pm$ 1σ_x	M $\pm$ 2σ_x	MEDIAN (FROM OGIVE)
Normals (N=100)	9.1	7.5	1.6 – 16.6	(–) 5.9 – 24.1	8.7
Hypoprothrombinemia (N=42)	18.2	12.4	5.8 – 30.6	(–) 6.6 – 43.0	15.0
Thrombocytopenia (N=35)	38.9	14.1	24.8 – 53.0	10.7 – 67.1	42.3

*Using three sampling errors as a criterion, all measures are statistically significant.

A further study of the structure of normal and abnormal blood clots was made by histologic examination after fixation in 10 per cent formalin in absolute alcohol. In the normal clot (Fig. 5) the erythrocytes are densely packed. A poorly retracting clot from a patient suffering from thrombocytopenia is demonstrated in Fig. 6, which shows loose packing of the erythrocytes, uniform fragmentation of the clot, and irregular inclusion of large pools of serum.

The extracorpuscular volume of the clot is composed of fibrin and unexpressed serum. Experimentally, we have determined that the volume occupied by fibrin in the normal recalcified plasma is equal to approximately 1 per cent of the total specimen.

DISCUSSION

Macfarlane suggested a relationship between a decrease in packed cell volume and a reduction in the volume of the clot when retraction is normal. However, he states: "This relationship does not apply when, in abnormal cases, retraction is deficient. In such cases retraction may be brought to a standstill not by the volume of the cells contained within the clot, but by an inherent inability of the fibrin to contract to the extent normally attained. Under these conditions it is clear that a reduction in the cell volume cannot increase the extent of retraction.

"Thus, whereas it is possible to correct by a simple calculation for the effect of anemia if retractility is normal, no such correction can be applied if it is

deficient. Hence the application of a correction to pathological cases will produce distortion, since deficient retraction will be revealed in anemic cases more often than in those with a normal cell volume."

It appears to us that blood clot retraction may be controlled through an equilibrium existing between the retractile force of the fibrin on the one hand, and the opposing resistance to aggregation of the retained cells on the other. Their relative influence may vary considerably, depending upon many factors, including the volume of the packed cells and the quantity and quality of the platelets, prothrombin, and fibrin. In the blood of normal subjects conditions appear to be fairly well balanced so that the average extracorpuscular volume of the clot is approximately 10 per cent of the original specimen. In the abnormal subject the same forces operate, but the balance may be upset because the retractile power of the fibrin may be diminished, and equilibrium is established when the average extracorpuscular volume of the clot is relatively large.

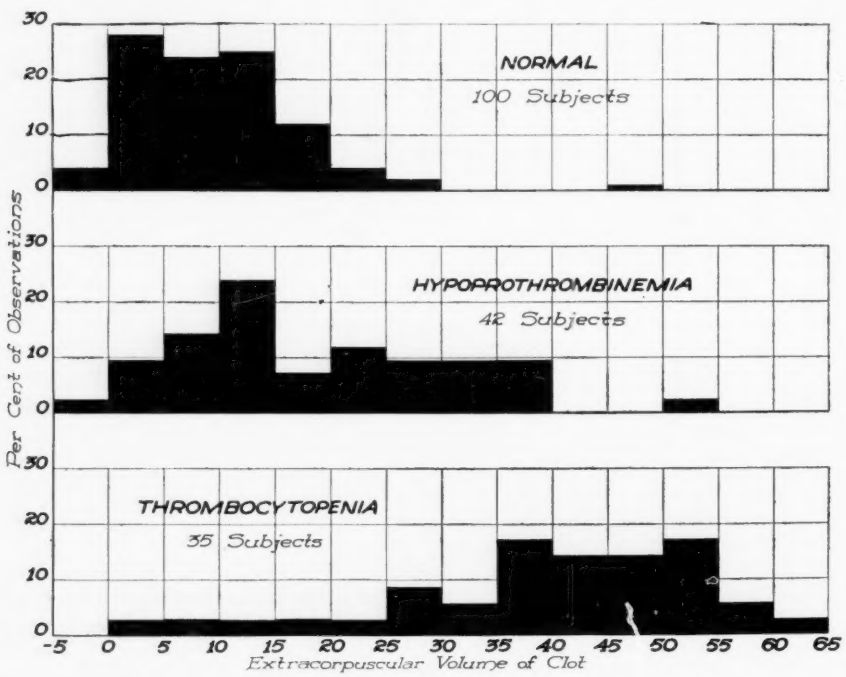

Fig. 4.—Frequency distribution of extracorpuscular volume of the blood clot in per cent of the total volume of the specimen.

When the packed cell volume is very low, and all other factors which influence clot retraction are normal, the maximum retractibility of fibrin may be achieved, and beyond this point there can be no further retraction regardless of the packed cell volume. It is obvious that this can happen only when clot retraction is excellent.

The volume occupied by the packed cells should be considered in estimating the degree of clot retraction. Failure to consider the effect of the cell volume on the volume of the clot may result not only in failure to detect deficient clot retraction in patients who have a normal cell volume, but also in the inability to recognize abnormal retraction in those who have severe anemia. Further-

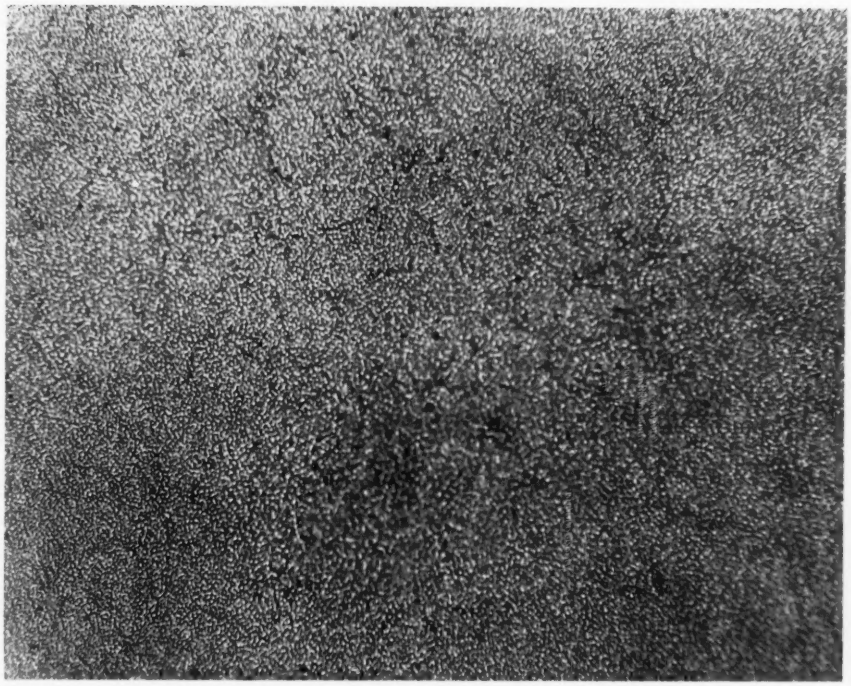

Fig. 5.—Section of a normal blood clot. Note the densely packed erythrocytes (×120).

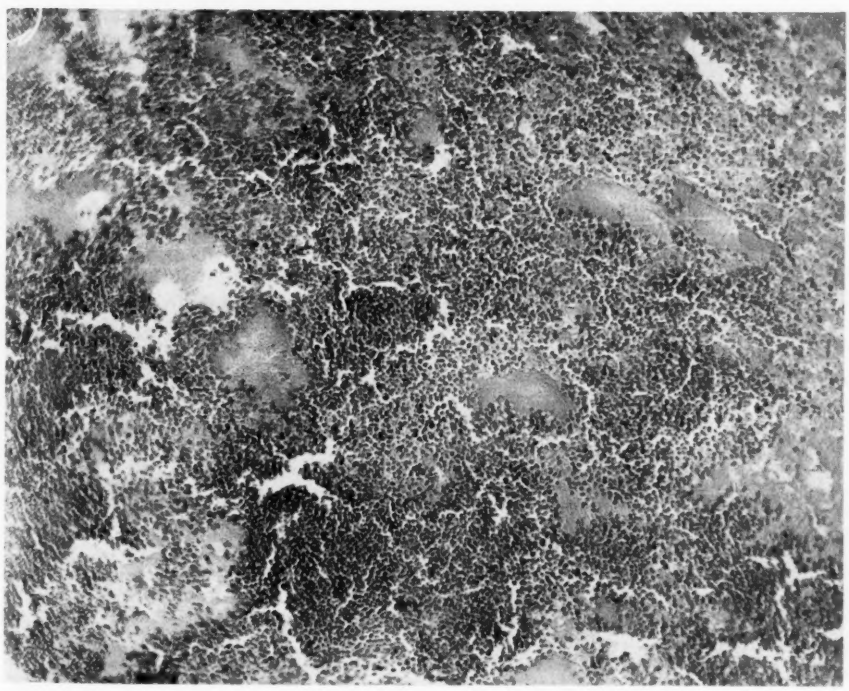

Fig. 6.—Section of a poorly retracting clot from a patient with thrombocytopenia. Note the fragmentation of the clot and the inclusion of large pools of fluid within the clot (×120).

more, unless the effect of the volume of the cells is appreciated, normal retraction may appear to be deficient in patients suffering from erythremia or leucemia.

In this study a highly significant relationship was found between diminished clot retraction and reduction in the platelet count. Tocantins[4] has made direct observations on the action of platelets in the mechanism of retraction of the blood clot. He states: "In the coagulation of blood from man and dog, soon after fibrin is laid down, *intact* platelets in the interior of the mass converge toward the fibrin needles, adhere to them and form large knots at their intersections. It appears that it is by thus strengthening the fibrin framework that platelets help to render the clot more rigid, firm and elastic. As the knots are being formed, the fibrin becomes bent, twisted and shortened. It is perhaps *while* this is going on and partly as a result of it, that the clot undergoes the visible reduction in volume (syneresis)." Furthermore, Tocantins[5] noted complete inhibition of blood clot retraction when antiplatelet serum was added to normal blood in vitro.

Two of us (P. M. A. and S. P. L.)[6, 7] have found that a significant correlation exists between the prothrombin concentration and the *clot retraction index* in patients suffering from obstructive jaundice and a variety of diseases affecting the liver. Boyce and McFetridge[1] noted that the prothrombin concentration was often diminished when the *serum volume index* was low. In the present study we have likewise observed in a significant number of patients suffering from a variety of diseases, an increase in the extracorpuscular volume of the clot when the prothrombin concentration was 50 per cent or lower. The reason for the frequent association of a diminished prothrombin concentration with deficient clot retraction may be due to incomplete conversion of fibrinogen to fibrin. The influence of the platelets on the degree of clot retraction is much greater than that of the prothrombin. It is possible that variations of the number of platelets within the normal range may be a factor in determining the degree of clot retraction, both in normal subjects and in those with hypoprothrombinemia.

Thrombocytopenia and hypoprothrombinemia are not the only factors concerned in clot retraction. We have observed patients in whom the prothrombin concentrations and the platelet counts were normal and in whom clot retraction was deficient; and conversely, we have studied patients in whom the prothrombin or platelet values were low and in whom the blood clots retracted normally. The recent observation of Rabinowitz[8] on the effect of amino acids on clot retraction may be of considerable aid in the solution of these problems.

SUMMARY

The blood clot is composed of cells, fibrin meshwork, and serum. The extracorpuscular volume of the clot is defined as that volume of the clot exclusive of its contained packed cells expressed as a percentage of the total volume of the specimen studied. Presumably, it is composed of serum occluded within the clot and a negligible amount of fibrin. The measurement of the extracorpuscular volume of the clot is a most significant factor in the analysis of the efficiency of blood clot retraction. In one hundred normal subjects the extracorpuscular mean volume measured 9.1 per cent and the standard deviation of this sample was 7.5 per cent; in 42 subjects who had prothrombin concentra-

tions of 50 per cent or less, the mean value was 18.2 per cent and the standard deviation was 12.4 per cent; and in 35 subjects, all of whom had platelet counts of 200,000 or less, the mean value was 38.9 per cent and the standard deviation of the sample was 14.1 per cent. We have arbitrarily considered the normal value to fall within two standard deviations of the mean (–5.9 to 24.1 per cent). Using this criterion, blood clot retraction was diminished in 13 of 42 patients who had hypoprothrombinemia, and in 30 of 35 patients who had thrombocytopenia.

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DRY BUFFER SALT MIXTURES FOR USE IN BIOLOGICAL PREPARATIONS*

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IN THE PREPARATION of many biological products the procedures specify the adjustment of a product to a given hydrogen-ion concentration. In many such procedures the accuracy of such an adjustment need not be great, provided the pH of the product does not exceed a given limit. Poorly buffered systems sometimes require time and considerable care to avoid exceeding the desired end point.

We have employed preparations of dry buffer salts which are useful, stable, and comparatively accurate. As we have not seen descriptions of dry salt preparations such as we use, and since these mixtures are very convenient, it seems worth while to describe them.

Sodium or potassium phosphate buffers were selected for pH values from 5 to 8. One per cent aqueous solutions of the salts selected for use were prepared and one solution was carefully titrated against the other in the presence of an indicator. The ratios of the buffer salts in solutions giving desired pH values were noted, and the amounts of the original dry buffer salts were determined. The solid buffer salts were powdered in a mortar, then weighed and mixed carefully in ratios determined by the titration, a different mixture

*From the Snodgras Laboratory of Pathology and Bacteriology, City Hospital, St. Louis. Received for publication, April 22, 1942.

being prepared for every pH value desired. For use, approximately 1 per cent of the dry buffer mixture of the required pH was dissolved in the product to be buffered.

In our work we wished to employ two buffer mixtures, one of pH 7.0 and the other of pH 7.6. Five cubic centimeters of 1 per cent $NaH_2PO_4.H_2O$ with 10 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH value of 7.0, and 5 c.c. of 1 per cent $NaH_2PO_4.H_2O$ with 40 c.c. of 1 per cent Na_2HPO_4 (anhydrous) gave a pH of 7.6. Thus one part of powdered $NaH_2PO_4.H_2O$ mixed with two parts of powdered Na_2HPO_4 (anhydrous) gave a mixture which when dissolved in water produced a pH 7.0. Likewise, one part of the former salt and eight parts of the latter was used for a mixture giving a pH 7.6. These mixtures were prepared in 500 Gm. lots and stored in tightly stoppered bottles.

Two examples may serve to show some of the ways we have employed these mixtures. In the culture of amoeba[1] it was found that the presence of buffers was necessary for successful propagation using the method described by Tsuchiya.[2] A simple method of producing small amounts of broth buffered at pH 7.0, was to add a loopful of the pH 7.0 dry buffer mixture to each culture tube whenever excessive acid was formed in the culture.

In preparing Price-type gonococcus antigen for the complement fixation reaction according to the directions of Torrey,[3] it is necessary to dissolve the trichloroacetic acid precipitated fraction in saline and sodium hydroxide. The final pH of the product should not exceed 9.5 or the antigenic value depreciates.[4] It is easier to prepare an active, stable product by adding a small amount of dry buffer mixture of pH 7.6 to a saline suspension of the precipitate than to add sodium hydroxide carefully drop by drop to the same end point.

The greatest convenience of the dry buffer salt mixture lies in its ability to produce sufficiently constant pH value with widely varying concentrations of the mixture. The constituents of the mixture should be powdered, accurately weighed, and carefully mixed. When the mixture has once been prepared, it is often sufficient to estimate roughly the amount of the mixture needed and to add the dry mixture to the product to be buffered directly from the stock bottle without weighing.

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A COMPARISON OF THE RYTZ, KAHN, AND COMPLEMENT FIXATION TESTS FOR SYPHILIS IN A LARGE SERIES OF ADULT MALES

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THE Rytz test is a rapid, economical precipitation method for the serologic diagnosis of syphilis. The purpose of this paper is to compare the results of 18,681 Rytz, standard Kahn, and complement fixation tests on 5,644 adult male patients, examined over a period of eighteen months, and to determine the accuracy and value of the tests. All case records were carefully reviewed.

The directions given by Rytz[1] were rigidly adhered to in the performance of this test, the Kahn[2] test was performed according to instructions, and the complement fixation test was performed according to the modification of Matz.[3]

For purpose of comparison the Rytz test is read positive or negative; the Kahn test as positive, doubtful, and negative; and the complement fixation test as positive and negative, as recommended by the Committee on the Evaluation of Serodiagnostic Tests for Syphilis of the United States Public Health Service, cooperating with the American Society of Clinical Pathologists.*

Of the 5,644 males tested 98.9 per cent were of the Caucasian race, and 1.1 per cent were colored. The ages ranged from 23 to 95, with an average of 48 years. Each patient received a Rytz, Kahn, and complement fixation test. Some patients received two or more tests, accounting for the total of 18,681 tests.

The patients were divided into four groups: group I: history and clinical evidence of syphilis with positive serology; group II: history and clinical evidence of syphilis with negative serology; group III: no history or clinical evidence of syphilis but with positive serology; group IV: no history or clinical evidence of syphilis with negative serology.

Group I: A total of 846 tests were performed on 149 patients. The Rytz test was positive in the case of 144 persons, or 96.6 per cent; the Kahn test in 137,† or 91.9 per cent; and the complement fixation test in 133, or 89.2 per cent. The Rytz test was positive and the complement fixation and Kahn tests were negative in one case; the Rytz test was negative and the complement fixation test was positive in 3 cases; the Kahn test was positive and the Rytz and complement fixation tests were negative in 2 cases; the Kahn test negative and the complement fixation and Rytz tests were positive in no instances; the complement fixation test was negative and the Kahn and Rytz tests were positive in 7 cases; and the Wassermann test was positive and the Kahn and Rytz tests were

*Complement fixation test: four plus, three plus, two plus, one plus, all recorded positive; plus-minus, doubtful. Kahn test: four plus, three plus, two plus, all recorded positive; one plus and plus-minus, doubtful.

†In addition, doubtful in 8 cases.

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negative in 3 cases. In only 6 cases, or 4 per cent of group I, is one test positive and the other two negative. It is evident that the Rytz and Kahn tests more closely agree than the precipitation tests and the complement fixation test.

In group II, 191 patients with history and clinical evidence of syphilis, many of them with previous records of positive serology, had received intensive antisyphilitic therapy and were discharged with seronegative findings, and at the time of this survey were negative serologically.

In group III careful study failed to elicit any history or clinical evidence of syphilis; however, 39 patients had positive serology by one or more of the three tests used. The Rytz test was positive in 27 patients; the Kahn test, positive in 19, doubtful in 7; and the complement fixation test, positive in 32. In this group again the results of the Kahn and Rytz tests were in close agreement, whereas the complement fixation was not in accord with the precipitation tests in 4 cases. The patients in this group had diagnoses of pneumonia, bronchitis, arthritis, appendicitis, undulant fever, duodenal ulcer, tuberculosis, pulmonary, renal calculus, and carcinoma. If the cases in this group are regarded as false-positive reactions, the percentage of the total number of patients tested is 0.4 per cent for the Rytz, 0.4 per cent for the Kahn, and 0.5 per cent for the complement fixation test.

In group IV, 5,265 patients gave no history or clinical findings of syphilis, and the serology was negative.

CONCLUSIONS

1. In a group of 149 syphilitic patients the Rytz test was positive in 96.6 per cent; the Kahn test, positive in 91.9 per cent and doubtful, or one plus, in 5.3 per cent; and the complement fixation test, positive in 89.2 per cent.

2. The so-called false-positive reactions in a group of 5,644 patients were 0.4 per cent for the Rytz, 0.4 per cent for the Kahn, and 0.5 per cent for the complement fixation test.

3. The Rytz test was found to give accurate results when compared with the Kahn and complement fixation tests and should be more widely recognized because of accuracy, speed, and economy in performance of tests for serodiagnosis of syphilis.

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BLOOD PLASMA PIPETTE*

W. Earl Mattox and John A. Leighty, Ph.D., Indianapolis, Ind.

IN THE course of our work of preparing large amounts of dried human plasma for the armed forces of the United States, it became necessary to develop a pipette for the separation of plasma from cells, which not only would be rapid, but also would give a high yield of plasma. In most pipettes used heretofore, it has not been possible to draw the plasma rapidly, and at the same time be able to remove it down to within a few millimeters of the junction between the cell layer and plasma layer without also pulling cells up with the plasma.

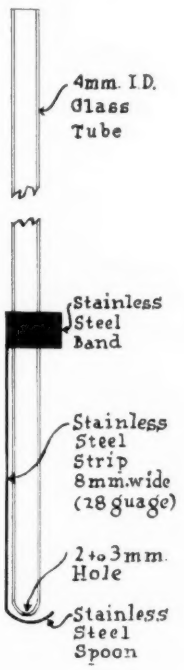

Fig. 1.

Fig. 1 shows a type of pipette which we are now finding very useful in large scale separation of plasma from cells. The stainless steel spoon and holder is made in one piece, and can be moved up and down on the glass tube. This pipette is most efficient when the end of the glass tube is just about touching the spoon.

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A TECHNIQUE FOR THE EVEN DISTRIBUTION OF GASES THROUGH BACTERIAL CULTURES*

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NEW YORK, N. Y.

NUMEROUS workers have noted the influence of various gases upon the growth of microorganisms. These include Wherry and Oliver[1] (1916), Chapin[2] (1918), Kohman[3] (1919), Reudiger[4] (1919), and Rockwell and McKhann[5] (1921), who observed that the growth of meningococci and gonococci was best when the medium was exposed to 10 per cent carbon dioxide. Novy, Roehm, and Soule[6] made a thorough investigation of microbic respiration (1925), and Novy and Soule[7] (1925) studied in detail the respiration of *B. tuberculosis*. Robertson and Sia[8] (1924) determined the growth-inhibitory and bactericidal action of sera and leucocytes upon pneumococcus at different rates of rotation of tubes during incubation. Soule[9] (1928) described the influence of hydrogen and nitrogen upon bacterial growth. Magoon and Brunstetter[10] (1930) devised a special apparatus for the bubbling of gas through media.

We arranged a relatively simple apparatus for the passing of gas through broth cultures held within a water bath. Four large test tubes (25 by 200 mm., 1 inch by 8 inches, 50 ml. capacity) were suspended in a constant temperature water bath.[11] This number was used because it was found most convenient for our studies on the effect of various concentrations of the sulfonamides at different temperatures on the growth of *Streptococcus viridans*. A smaller or larger number could have been employed as well. Each tube, containing 30 c.c. of a 0.4 per cent glucose infusion broth with a pH 7.4, was inoculated with 1 c.c. of an eighteen-hour-old broth culture of *Streptococcus viridans*. The cultures maintained were kept at temperatures ranging from 37.5° C. to 41.5° C. The temperature of the water bath was held constant to within ± 0.05° C. by heaters, thermostat, and stirrer.

After a series of trials a gas mixture, consisting of 10 per cent carbon dioxide, 5 per cent oxygen, and 85 per cent nitrogen, was found favorable for the growth of *Streptococcus viridans*.† A tank containing these gases was connected to a regulator with a pressure gauge and a four-way outlet. From each of these outlets the gases flowed through separate wash bottles to the culture tubes. After bubbling through the cultures, the gas was permitted to pass through another series of wash bottles and then into the air. The constant slow agitation of the culture medium prevented the settling of the bacteria on the

*From the Laboratories of Bacteriology and Physical Therapy, the Mount Sinai Hospital, New York.

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†A similar mixture was employed by Longsworth and MacInnes[12] in studies on the growth of *Lactobacillus acidophilus* at varying pH and temperature ranges.

bottom of the tube and insured an equal exposure of the organisms to the surrounding environment. The gas within the culture tube coming from the tank as well as that due to the metabolic activity of the organisms, passed continuously through the sidearm into the exhaust gas bottle.

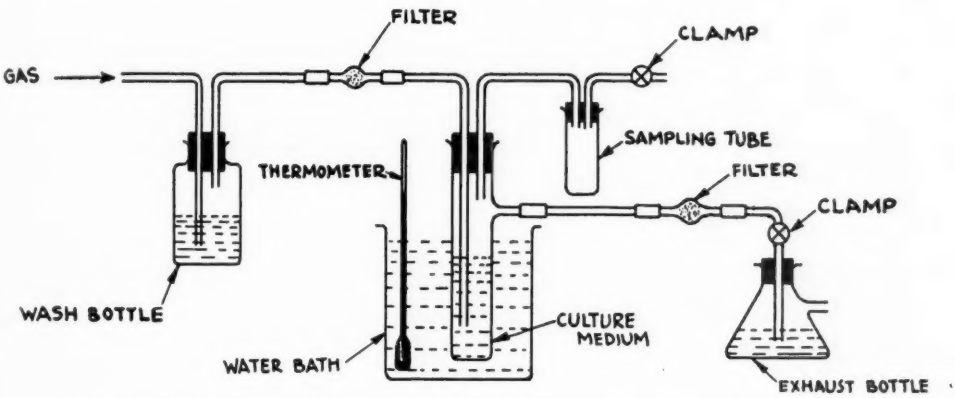

Fig. 1.—Gas coming from the tank passes through a wash bottle, then through a filter into the culture medium. The tube containing the medium is immersed in a water bath, the temperature of which is held constant by heating units, thermostat, and stirrer. The gas passes from the tube containing the culture medium through a side opening into an exhaust wash bottle from which it goes into the air. Closing the clamp on the tube going to the exhaust wash bottle causes the pressure within the culture tube to be raised, forcing the medium through another opening into the sampling tube. After the desired amount of culture medium collects in the sampling tube, the clamp on the tubing coming from it is closed, and the clamp on the tube going to the exhaust bottle is opened to re-establish the previous course of the flow of gas.

When it was necessary to remove a sample of the culture, the opening to an exhaust gas bottle was closed. This caused the building up of gas pressure within the culture tube which forced some of the medium through another opening leading to a sterile sampling tube. A clamp on the opening leading from this tube controlled the inflow. After the desired amount of medium had accumulated in the sampling tube, the outlet from this tube was shut off and the clamp restricting the flow of air into the exhaust bottle was opened.

This technique permitted the removal of the culture sample without contamination of the medium in the large culture tubes. Following the removal of the sample, another empty tube was substituted for it. The sample was thoroughly diluted in 0.04 per cent glucose infusion broth 10^{-6} and $10.^{-7}$ Poured blood agar plates were then made with the sample variously diluted for bacterial counts.

SUMMARY

A technique is described for the cultivation of microorganisms under the following conditions, namely: (a) constant slow agitation of the cultures during the entire period of growth; (b) carefully controlled temperature; (c) an even distribution of mixture of gases favorable for the microorganisms in the media; and finally, (d) easy removal of samples without any danger of contaminating the culture at any time during the cultivation.

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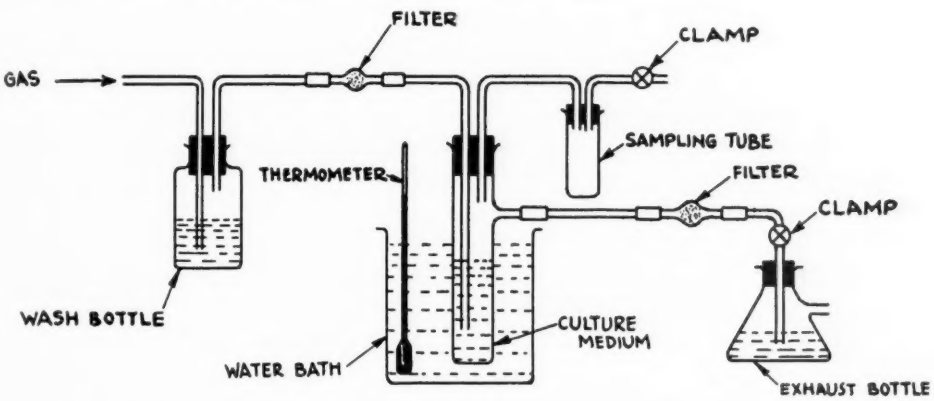

Fig. 1.—Gas coming from the tank passes through a wash bottle, then through a filter into the culture medium. The tube containing the medium is immersed in a water bath, the temperature of which is held constant by heating units, thermostat, and stirrer. The gas passes from the tube containing the culture medium through a side opening into an exhaust wash bottle from which it goes into the air. Closing the clamp on the tube going to the exhaust wash bottle causes the pressure within the culture tube to be raised, forcing the medium through another opening into the sampling tube. After the desired amount of culture medium collects in the sampling tube, the clamp on the tubing coming from it is closed, and the clamp on the tube going to the exhaust bottle is opened to re-establish the previous course of the flow of gas.

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CHEMICAL

RELATION OF THE CONCENTRATION OF STARCH SUSPENSIONS TO THEIR USE AS SUBSTRATES FOR THE AMYLASE OF THE DUODENAL CONTENTS OF INFANTS*

WILBURT C. DAVISON, M.D., DURHAM, N. C.

IN STUDYING the effect of the amylase of the duodenal contents of infants on the viscosity of starch suspensions in tenth-molar phosphate mixtures,† the successive measurements of viscosity must be plotted in order to determine the rate of change, since the time required to produce a definite amount of change was inversely proportional to enzymic activity; e.g., if 0.1 c.c. of an enzyme preparation required sixty minutes to reduce the initial viscosity 20 per cent, 0.2 c.c. reduced it a similar amount in thirty minutes.[1] On the other hand, the amounts of change in a definite time were not proportional; e.g., regardless of the strength of the enzyme, the viscosity of starch suspensions to which different amounts of amylase had been added, usually but not always, fell to that of the phosphate mixture in two to twelve hours, and then rose nearly to its original viscosity. This secondary increase in viscosity may be due to the viscosity of the end products of starch digestion, e.g., amylocellulose.

As the strength of an enzyme was inversely proportional to the *time* required to effect a definite percentage of change in the viscosity of the substrate but not to the *amount* of change in a definite period of time, an amylase unit was defined as that amount of enzyme which required sixty minutes to reduce the initial viscosity 20 per cent.[1] Sixty minutes and a 20 per cent change were used to define a unit since faster rates were difficult to determine accurately because the frequent measurements of viscosity consumed one to two minutes each, and the enzymic activity was continuing during the observations; slower rates allowed bacterial growth and other sources of errors to occur. Side reactions, which affected the viscosity, occurred with changes greater than 20 per cent in the substrate.

This linear relationship between the velocity of an enzymic reaction and the amount of enzyme used holds only when the amount of enzyme is much smaller than that of the substrate, so that all of it can combine with the latter.[2, 3] With a relative decrease in the concentration of the substrate, the velocity may be proportional to the square root of the concentration of the enzyme, the so-called Schütz and Borissov law,[4] e.g., increasing the concentration of sucrose up to 4 or 5 per cent increases the velocity of invertase reactions,[5] but concentrations

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†All of these studies were made with the same preparation of soluble starch (Merck; made according to Lintner.

above that level decrease the rate of hydrolysis.[6] With colloidal substrates, as in the action of trypsin on gelatin, an increase in the concentration of the substrate also reduces the rate of hydrolysis, due either to the increased viscosity or to the products of hydrolysis.[3]

Viscosity determinations in enzymic measurements can be plotted against time in four ways: (1) As the "observed" viscosity (η, the outflow time of 5 c.c. of the starch-enzyme mixture); (2) the "net" viscosity (η-η_o, the outflow time of the buffer solution), (3) the "relative" viscosity (η/η_o), and (4) the "specific fluidity" (η_o/η-1). The resulting curves have the same general shape, and since the first method is simpler and as accurate as the other three, it was used in routine amylase titrations in infants.[7] As several viscosimeters were used in these titrations, the initial "observed" viscosities frequently were different,[8] and, therefore, were expressed as 100 per cent, and the subsequent measurements as percentages of the initial "observed" viscosity.

TABLE I

EFFECT OF THE CONCENTRATION OF STARCH SUSPENSIONS ON THE MEASUREMENT OF AMYLASE, BASED ON THE PERCENTAGE CHANGE IN THE "OBSERVED" VISCOSITY (FIG. 1)

CONCENTRATION OF THE STARCH SUSPENSIONS (%)	OUTFLOW TIME OF 5 C.C. OF THE STARCH SUSPENSIONS (η SEC.)	OUTFLOW TIME OF 5 C.C. OF THE BUFFER SOLUTION (η_o SEC.)	RELATIVE VISCOSITY η/η_o	TIME REQUIRED FOR A 20% REDUCTION IN THE INITIAL "OBSERVED" VISCOSITY (SEC.)	AMYLASE UNITS*
7.5	385.2	33.0	11.67	16.5	3.6
7.5	351.4	29.5	11.91	16.5	3.6
5.0	257.7	49.7	5.18	10.5	5.6
5.0	248.0	48.0	5.17	10.5	5.6
2.5	90.5	41.3	2.19	19.0	3.2
2.5	127.4	55.8	2.28	19.0	3.2
1.04	39.2	28.5	1.38	∞	0
1.04	40.6	29.5	1.37	∞	0

*The amount of enzyme which requires sixty minutes to reduce the initial viscosity 20 per cent.[1]

As shown in Table I and Fig. 1, in which the percentage changes in the "observed" viscosity were plotted against time, the addition of the same amount of duodenal contents to starch suspensions of different concentrations produced varied results. In the 1.04 per cent starch suspension the enzymic activity was similar to that in the other concentrations for twenty minutes and then stopped; agglutination occurred at the end of two hours. The rate of the enzymic hydrolysis of 2.5, 5.0, and 7.5 per cent starch suspensions was similar for the first forty minutes, but then the rate of enzymic activity in 2.5 per cent starch practically ceased. The rate of hydrolysis of 5 per cent starch was somewhat faster than that of 7.5 per cent starch for the first one hundred minutes and then became slower. As shown in Fig. 1, the solid and open points of the parallel titrations practically coincided.

After twenty-four hours at 34° C. all the starch-duodenal contents mixtures became agglutinated and their viscosity could not be determined. Some undigested starch remained in the 5 and 7.5 per cent suspensions but not in the

1.04 and 2.5 per cent suspensions after twenty-four hours; i.e., the addition of 5 c.c. of 1:62.5 Gram's iodine to 1 c.c. of the starch-duodenal contents mixtures produced a purplish blue color in the two former, but no color in the last two. All the substrates turned blue when the iodine solution was added before incubation with the duodenal contents.

As the times required for a 20 per cent reduction in the "observed" viscosity of 2.5, 5.0, and 7.5 per cent starch concentrations were more closely related than that for the hydrolysis of 1.04 per cent starch, the higher concentrations were used in other viscometric measurements of amylase.[7] A suspension with a relative viscosity of approximately 2.5 (Table I) was the easiest to work with as clogging of the viscosimeters occurred frequently with the heavier suspensions. Furthermore, the 7.5 per cent suspension became caramelized when autoclaved.

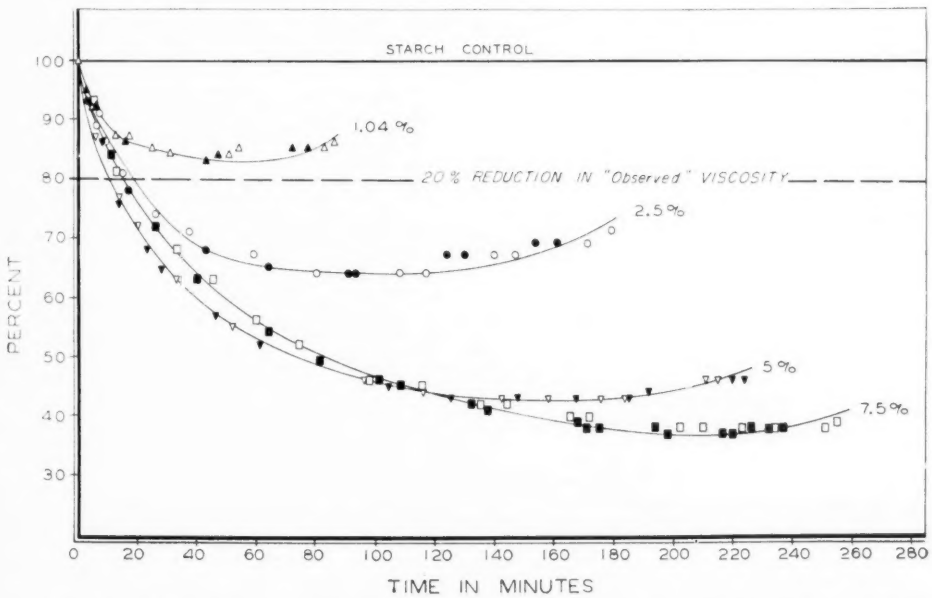

Fig. 1.—Effect of the concentration of starch suspensions on the measurement of amylase, based on the "observed" viscosity. One-tenth cubic centimeter of the same specimen of duodenal contents (diluted 1:20 with normal saline) was added to 10 c.c. of different starch concentrations, and the amylase activity was titrated viscometrically[1] in duplicate. The initial outflow times of the starch-duodenal contents mixtures in seconds (Table I) were plotted as 100 per cent (initial "observed" viscosity), and subsequent measurements as percentages of this initial "observed" viscosity. One of each pair of parallel titrations was plotted in solid points, and the other in open.

The relative viscosity of starch suspensions varied with the particular lot of soluble starch employed. A 2.5 per cent suspension of the starch used in Table I produced a substrate of this desired "observed" viscosity, but as much as 6 per cent of other samples of starch was required to produce a suspension of similar viscosity.[8] The digestibility of different lots of starch also has been found to vary, probably due to differences in the proportions of α- and β-amyloses and dextrins.[9] More uniform results have been reported by the use of substrates consisting of mixtures of two preparations of starch.[10]

The viscosity of starch suspensions made with cold phosphate mixtures was fairly constant, but boiling them, or preferably autoclaving them, for fifteen

minutes at 15 pounds pressure made the results uniform. The maximum viscosity was obtained by heating starch solutions to 93° C. Higher temperatures reduced the viscosity. However, starch suspensions, which were both boiled and autoclaved, were used routinely because they were more homogeneous and less likely to contain small lumps which clogged the capillary of the Ostwald viscosimeter, than those which were merely heated to 93° C. or boiled or autoclaved. Amylase titrations with boiled or autoclaved starch suspensions were similar.

The viscosity of autoclaved suspensions (without enzymes) of 1.04, 2.5, and 5 per cent starch was constant for twelve hours, but that of 7.5 per cent starch increased steadily for two hours and then remained constant for twelve hours. After twelve hours the viscosity of all the starch suspensions (without enzymes) increased, probably due to an increase in amylocellulose. However, if these starch suspensions were centrifuged after eight days, the viscosity of the supernatant fluid was the same as that of the phosphate mixture, suggesting that no more of the starch had dissolved. Occasionally a preparation was found which decreased in viscosity. Such suspensions, of course, were useless for amylase determinations.

Amylase determinations, employing starch suspensions less than twelve hours old, similarly made with the same preparation of Lintner starch on different days, were comparable, though far from accurate. Irregular results were obtained with older substrates, even though thymol was added to insure sterility. For example, the rate at which duodenal amylase produced a 10 per cent reduction in the viscosity of fresh and of nine-day-old starch suspensions was the same, but the time required to cause a 20 per cent reduction was much longer with the older preparation than with the fresh suspension.

The starch in these suspensions, made with Lintner soluble starch, apparently consisted of microscopically visible starch granules and ultramicroscopic colloidal particles. The latter probably represented the fraction attacked by amylase, for after the enzyme had reduced the initial viscosity of a starch suspension until it was practically the same as that of the phosphate mixture, the number of starch granules was not reduced. Furthermore, if a starch suspension which had been split by amylase was allowed to stand a few hours, the starch granules sank to the bottom of the tube, leaving the supernatant fluid water clear, in contrast to the supernatant fluid of an unsplit suspension which remained opalescent indefinitely, even though diluted with tenth-molar phosphate mixture until its viscosity was the same as that of the starch suspension which was split by amylase. Because of these two types of starch, it was not possible to determine the actual starch content of starch suspensions at any stage in enzymic reactions by merely comparing their viscosity curves with those of suspensions of unsplit starch of known concentrations.[8]

CONCLUSION

A starch suspension with a relative viscosity of approximately 2.5 and less than twelve hours old was the most suitable for the viscometric titration of the amylase of the duodenal contents of infants, but the results were not always accurate. However, viscometry[1] is preferable to other methods.[13]

Sufficient soluble starch (Merck; made according to Lintner) to make a suspension with a relative viscosity of approximately 2.5 (2 to 6 Gm., depending on the preparation) should be added to 100 c.c. of tenth-molar phosphate mixture (40 c.c. M./10 KH_2PO_4 + 60 c.c. M./10 Na_2HPO_4). The suspension should be shaken vigorously until it becomes homogeneous (usually one minute), boiled for one to two minutes, and autoclaved for fifteen minutes at 15 pounds pressure; one or two thymol crystals, should be added, and the suspension filtered, while hot, through one thickness of muslin. After it has cooled to about 34° C. 10 c.c. portions should be pipetted into clean test tubes and kept in the water bath for at least one hour and not more than twelve hours before being used. The final reaction of this substrate is pH 6.8 to 7.0, which is optimal for the amylase of the duodenal contents of infants.[11] If the amylase of taka-diastase is being titrated, phosphate mixtures of pH 3.0 to 4.8 should be used.[12] All the titrations should be done with the same lot of soluble starch. Other concentrations of starch have been used and various methods of preparation tried, but the above appeared to be the most satisfactory.

I wish to thank Dr. Elizabeth Torrey (Mrs. J. G. Andrews) for plotting the enzyme curves and calculating the units; Mrs. H. A. King and Mr. E. H. Clark for the drawings; Dr. Hans Neurath, Dr. Paul Gross, Dr. D. G. Hill, Mr. J. H. Neese, and Mr. W. V. Singletary for verifying the calculations.

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A METHOD FOR THE DETERMINATION OF CERTAIN SULFONAMIDES IN BILE*

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WITH the increasing use of sulfonamide compounds in the treatment of infection, a knowledge of the excretion of these drugs in the bile has assumed greater importance. A method for determination of sulfanilamide in bile was presented by Ivy and one of us (H. M. C.)[1]; this was a modification of the method of Doubilet. With the recent refinements[2] in chemical procedures employed in the determination of sulfanilamide, and with the introduction clinically of several new drugs in this field, it seems advisable to extend the previously published method in order to include the new improvements and to test the applicability of the method to the new drugs.

We have found the following procedure satisfactory for the quantitative determination of sulfanilamide, sulfapyridine, sulfadiazine, and sulfathiazole in bile. With the last-named drug an additional step must be used.

METHOD

Place 5 c.c. of the bile to be examined in a 50 c.c. volumetric flask. If concentrations of the sulfonamide compound of 2 mg. per 100 c.c. or less are expected, use 10 c.c. of bile. Add 3 c.c. of 2 N potassium hydroxide. Add 3 c.c. of a 40 per cent solution of zinc sulfate drop by drop, with constant agitation. Add portions of hot water and agitate the precipitate to insure solution of the drug. Dilute to approximately 45 c.c. Then add 3 c.c. of normal potassium carbonate. Add a drop of phenolphthalein indicator solution. Dilute when cool to exactly 50 c.c. Filter. Acidify the filtrate exactly to phenolphthalein by the addition of 2 or 3 drops of 4 N hydrochloric acid. By acidifying after filtration, pigmented substances are eliminated by filtration and do not go into solution as they would if the acid were added before they are removed. Dilution of filtrate by acid solution may be considered negligible.

To 10 c.c. of the filtrate add 1 c.c. of tenth-normal hydrochloric acid. Agitate for a moment until no more effervescence is noted. This will prevent interference from bubbles in the photelometer cups in a later stage. Then add 1 c.c. of a freshly prepared 0.1 per cent solution of sodium nitrite. Agitate and allow to stand for three minutes. Add 1 c.c. of a 0.5 per cent solution of ammonium sulfamate. Agitate and allow to stand for two minutes. Add 1 c.c. of a 0.4 per cent aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride. The color developed is read immediately against simultaneously prepared aqueous standards in a colorimeter or, if facilities are available, with a photelometer equipped with a filter.

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When sulfathiazole is the drug to be determined, a slight modification of the method is necessary because of the insolubility of the dye product formed. It is necessary to add 5 c.c. of ethyl alcohol to the 10 c.c. portion of filtrate and to the 10 c.c. portion of the standard solution for comparison in the colorimeter before addition of the solution of sodium nitrite. This will insure the solution of dye in subsequent procedures.

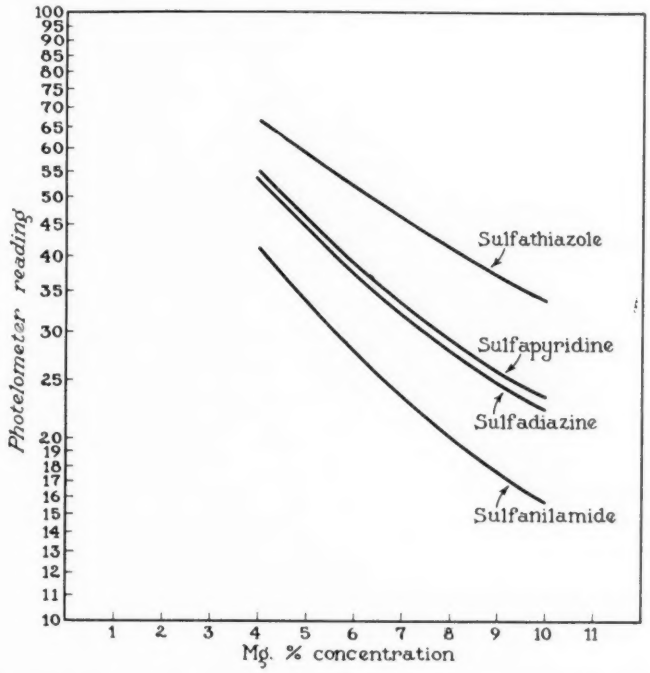

Fig. 1.—Relationship of concentrations of sulfonamides and readings on the photelometer.

This method as described will determine only the drug in its free form; acetylated drug must be hydrolyzed if the total content of drugs is to be found. If this is desired, it is necessary to add to the 10 c.c. of filtrate 0.5 c.c. of 4 N hydrochloric acid and place in a bath of boiling water for one hour. After cooling, make the volume 10 c.c. Proceed as with 10 c.c. of original filtrate with the method described.

Should the colorimeter be used in this determination, the following formula may be employed:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{strength of standard}$$

that is, 0.06 mg. × 100 = concentration of unknown in milligrams per 100 c.c.

We have found the photelometer accurate in this determination when a green filter allowing maximal transmission of 530 Å units is employed. As the dye produced in the reaction has a maximal absorption of 545 Å units,[2] a considerable amount of extraneous color is eliminated suitably. When this filter is employed, the error introduced by the use of aqueous standard rather than bile filtrate will average 1.8 per cent and will not exceed 3 per cent.

Bile which was known to contain 10 mg. per 100 c.c. of the afore-mentioned sulfonamide compounds was used to test the accuracy of the method (Table I). The method is more accurate when sulfanilamide, sulfapyridine, or sulfadiazine is present in the bile than when sulfathiazole is present.

TABLE I

RECOVERY OF SULFONAMIDE COMPOUNDS FROM BILE IN WHICH THE CONCENTRATION WAS KNOWN TO BE 10 MG. PER 100 C.C.

SAMPLE	MILLIGRAMS PER 100 C.C.			
	SULFANILAMIDE	SULFAPYRIDINE	SULFADIAZINE	SULFATHIAZOLE
1	9.7	9.3	9.6	7.7
2	10.0	10.1	10.2	8.1
3	9.7	9.9	9.8	7.8
4	9.5	9.3	9.8	7.3
5	9.7	9.3	9.9	8.0
6				8.5
7				7.8
8				8.1
9				8.0
10				8.6
Average	9.7	9.6	9.8	8.0

The curves from which these data were read are given in Fig. 1. Because of a slight variation in photoelectric cells in these photelometers, a curve should be established for each instrument.

An attempt was made to determine the accuracy of photelometric curves for the sulfonamides on the semilogarithmic graph. By introducing factors to correct for differences in dilution, as in the case of sulfathiazole, and for differences in molecular weight, identical curves should be established. An arbitrarily selected intensity of color was chosen on an abscissa of the graph which was intersected by all curves plotted. Log 4.00 was chosen. The concentration of the drugs at this level of color intensity was then converted by the afore-mentioned factors to terms of concentration of sulfanilamide.

The concentration of all drugs when converted to terms of sulfanilamide and read at the color intensity of log 4.00 varied only slightly. The concentration thus obtained was 3.99 mg. per 100 c.c. $\pm$ 0.17 mg. per 100 c.c.

SUMMARY

A method has been presented by which the concentration of sulfanilamide, sulfapyridine, sulfadiazine, or sulfathiazole in bile may be determined. This method has been tested with solutions of known concentration and percentage rates of recovery have been determined.

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AN OSMOMETER FOR USE WITH SERUM AND ITS SUBSTITUTES*

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THE present use of "banked blood," stored or dried plasma, and plasma substitutes in the treatment of shock from trauma, burns, or hemorrhage has as its aim the restoration and maintenance of normal blood volume. This aim is accomplished by the injection of solutions which contain, not merely salts and sugars, but also some material which produces a sufficient "protein osmotic" or "oncotic" pressure (erroneously called "colloid osmotic pressure"†) approximately equal to that of normal plasma.

In the case of serum or plasma the "protein osmotic" pressure can be estimated by calculations based on the content of albumin and of globulin. The albumin is responsible for about 80 per cent of the pressure. A rough approximation can be made also from the protein content, as determined by protein nitrogen or plasma specific gravity. However, with dried plasma, gelatin, pectin, or other blood substitutes, the osmotic pressure can best be determined by direct measurement.

The use of a long tube attached to a membrane[1] requires twenty-four hours or longer to reach equilibrium, so that bacterial action and dilution of the serum can cause serious errors. Turner[2] in 1932 devised a method for counterbalancing the pressure so as to reach equilibrium in five to twenty hours. In 1936 Sumalt and Landis[3] constructed an ingenious, but complicated, apparatus which automatically counterbalanced and recorded the pressure until equilibrium was reached. Hepp[4] in 1936 invented a micro-osmometer in which ultrafiltrate from a sample of serum was sucked through a large membrane into a capillary. The balancing pressure was then adjusted until the capillary meniscus was seen through a microscope to be stationary. Peters and Saslow[5] claimed to obtain good results with this osmometer of Hepp. There is, however, the danger that

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†We dislike all three of these terms which are applied to the osmotic pressure observed when a nondiffusible ion on one side of a membrane is in equilibrium with diffusible inorganic electrolytes. It was stated by Peters and Van Slyke (Quantitative Clinical Chemistry, Vol. I, page 659) that of the 25 mm. (of mercury) osmotic pressure exerted by blood plasma against isotonic saline solutions, 20 mm. pressure is due to the protein molecules while 5 mm. pressure results from the unequal distribution of diffusible ions according to Donnan's law (Zschr. f. Electrochemie **17**: 572, 1911). The latter phenomenon was further studied by Jacques Loeb (J. Gen. Physiol. **3**: 667, 691, 827, 1920; **4**: 73, 1921).

The term "protein osmotic pressure" is applicable only to systems, such as plasma, where the nondiffusible ions are proteins. The term "colloid osmotic pressure" is particularly objectionable, since it implies that the pressure involves a colloidal phenomenon. Actually the large ion need not be a protein (Donnan used a dye). Perhaps "oncotic pressure" is the least objectionable term in current usage which is applied to the phenomenon. However, this connotes "swelling" and does not distinguish, except by usage, between the observed osmotic pressure when only the large ions are nondiffusible and the theoretical osmotic pressure due to all ions and molecules. We prefer the term "Donnan pressure" but realize that this also is not clearly defined.

the large capillary correction (about 90 mm. of water) and also the use of rubber connection may cause appreciable errors. The errors of the Krogh apparatus were discussed by Wells.[6]

The apparatus herewith described (Fig. 1) was designed to give rapid and accurate results which involve no capillary correction. It uses only 1 to 2 ml. of serum or other fluid, and gives accurate readings within fifteen minutes.

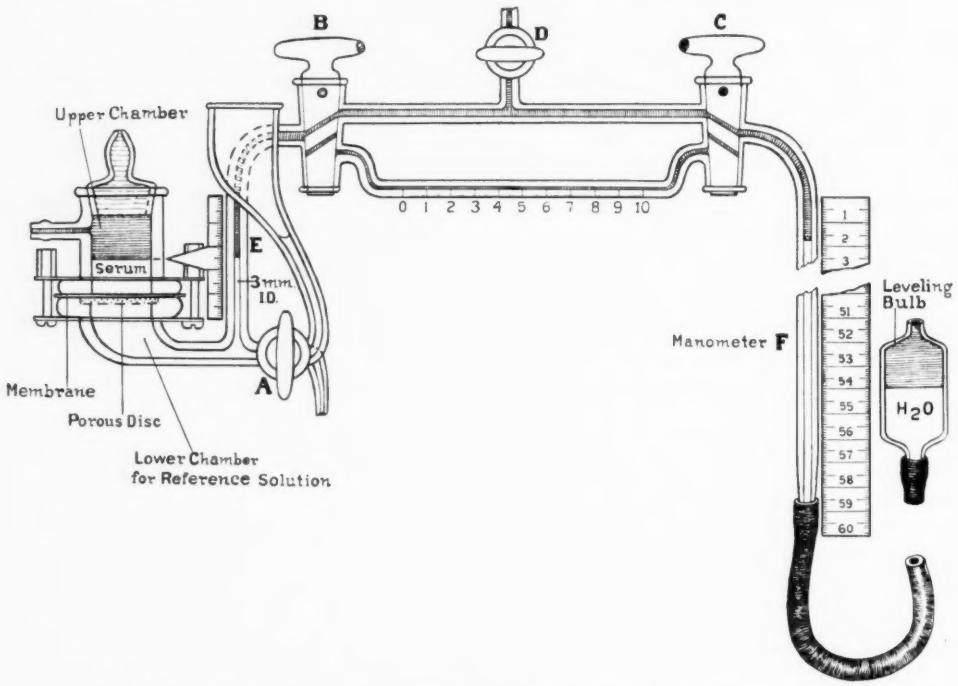

Fig. 1.—The S.Z.L. osmometer. This is firmly supported on a wooden stand not shown in the diagram. Air spaces are shaded, solutions are shown white and the kerosene drop is indicated in black. Detailed description is in the text. The centimeter divisions on the two scales may serve to indicate the dimensions of the apparatus.

DESCRIPTION OF OSMOMETER

In Fig. 1 the osmometer may be seen to consist of the following parts: a removable upper chamber, for the serum or other solution, with an inside diameter of 25 mm. The side arm and the hole in the cover permit the flow of carbon dioxide—air mixture over the solution to maintain pH. The upper chamber is clamped to a lower chamber with the membrane in between glass surfaces which are ground flat. The membrane is supported by a porous disk of fritted pyrex or alundum. The lower chamber is used for the "reference fluid" (such as serum ultrafiltrate or similar physiologic solution), which is introduced from a filling cup through stopcock *A*. The same stopcock serves to drain the chamber. The lower chamber is connected through tube *E* and stopcocks *B* and *C* to the manometer tube *F*, to which a small leveling bulb is attached with heavy walled 1/8 inch rubber tubing. Tubes *E* and *F* should have the same inside diameter (about 3 mm.). Stopcocks *B* and *C* are connected by an upper, 2 mm. bore, tube (having an outside stopcock *D*) and also by a lower capillary tube

in which a drop of colored kerosene serves to indicate motion. The membrane area is 1,000 to 1,700 times the capillary cross section and an observable movement of 0.2 mm. in the capillary represents a displacement of only 0.003 to 0.005 per cent of the serum volume.

METHOD FOR USING OSMOMETER

The osmometer is prepared for use by first cleaning it and then attaching it to a firm board. The leveling bulb is filled with distilled water and is supported by an adjustable clamp. A small drop of kerosene, colored with scarlet red, is introduced into the horizontal capillary tube by removing the plug of stopcock *B* and using a syringe with a bent needle or a dropper with a fine bent tip. By lowering the leveling bulb slowly, the drop is drawn into the tube; it should be 5 to 10 mm. long. No kerosene should remain at the end of the tube near the stopcock.

To fill the osmometer for the zero reading, the porous disk is put in place and the lower chamber is filled with the desired "reference fluid," such as serum ultrafiltrate[7] or a physiologic solution such as is used in these laboratories.[7] This "reference fluid" is introduced from the filling cup through stopcock *A* while stopcocks *B* and *D* are open. A prepared collodion membrane (see below) is then placed over the disk in such a manner as to exclude bubbles of air. The upper chamber is clamped in position and into it 2 or 3 ml. of the "reference fluid" is placed. With *B* and *C* open across the upper tube (and *D* closed) the leveling bulb is lowered to give a negative pressure of about 200 mm. of water for two or three minutes. This pulls the membrane down tight onto the disk. Then stopcock *B* is closed and *D* is opened. The leveling bulb is then raised to bring the water near the top of the manometer tube *F* and stopcock *D* is closed. The "reference fluid" in the vertical tube *E* should be at approximately the level of the fluid in the upper chamber. It can be adjusted by stopcock *A*.

A "zero reading" is taken by opening stopcock *B* and *C* to the lower (capillary) tube and then adjusting the leveling bulb until the drop of kerosene remains stationary for five minutes. If the drop moves to the right, the bulb should be raised. If it moves to the left, the bulb should be lowered. A half minute should elapse after each adjustment before the first observation is made, and the next reading is taken at least a minute later. With a little practice the amount of adjustment required can be estimated from the rate of movement of the kerosene drop. When there has been no movement for five minutes, a record is made of the height of the water column in the manometer above the water surface in the leveling bulb. The height of the "reference fluid" in tube *E above* the fluid surface in the upper chamber is also recorded and *subtracted* from manometer *F* reading to obtain correct "zero reading." If the fluid in *E* is below that in the upper chamber, the difference is added to the manometer reading.

The zero reading should equal zero. Any deviations may be due to an air bubble under the membrane, or a failure to draw the membrane down flat against the disk, or to kerosene at an end of the capillary tube. The zero reading should be repeated after momentarily reversing stopcocks *B* and *C*

and, if desired, readjusting the water level while *D* is open. With the pressure set at the previous reading there should be no movement of the kerosene drop.

Readings on the unknown solutions are taken next. Stopcock *B* is closed, the fluid in the upper chamber is drawn off, and the membrane is dried quickly with filter paper. One to 2 ml. of the serum or other unknown solution is introduced immediately. *B* and *C* are opened to the upper tube, and the leveling bulb is lowered to approximately the expected pressure. Adjustments are then made, as described above, preferably alternating the adjustments above and below the final reading, thus making sure that there is a real Donnan pressure. When the kerosene drop remains stationary for at least five minutes, the manometer is read. It can be checked by repetition of the procedure.

The membrane can be used for a number of unknown solutions, each solution being removed and the membrane wiped with filter paper before the next solution is placed in the upper chamber. It is well to start with the more dilute solutions and finish with the more concentrated solutions, or else wash the membrane with the "reference fluid" between samples.

Cleaning of the osmometer after use can be done with cleaning solution, which is allowed to stand in the filling cup and lower chamber and is drawn up into tube *E*. There is less danger of affecting the pH if 0.01 molar sodium hydroxide is used, instead of dichromate cleaning solution, and followed by 0.02 molar acetic acid, rinsing with distilled water after each solution.

Membranes. We obtained best results with membranes made from Merck's C.P. 5 per cent collodion solution on machines previously described.[7] These tubular membranes are cut into 50 to 55 mm. squares or circles and are preserved under distilled water, never being allowed to dry out. For twenty-four hours or more before use, the membranes are allowed to soak in physiologic solution (preferably "Z solution"[7]). This insures saturation of the membranes with calcium and permits a rapid equilibrium which is not otherwise possible. The Viscose Corporation sausage skin and No. 600 DuPont cellophane also gave good results, but these have a pore size of about 2 mμ, which may account for the longer time necessary for a detectable movement of the kerosene bead to appear.

Flat membranes, similar to the first type but less satisfactory, were made by pouring the collodion solution onto mercury in a Petri or similar dish. One milliliter of collodion solution covers 6.5 to 7 sq. cm. of surface. Hence a dish with a diameter of d cm. will require 0.12 d^2 ml. Allow to dry for thirty minutes in a dust-free atmosphere, soak in 50 per cent alcohol for fifteen minutes or longer, then wash with distilled water.

Pore size of the membranes. The osmometer can be used for the measurement of alleged pore size of membranes, as it is commonly rated. Distilled water is placed in both upper and lower chambers, using a membrane which has soaked in distilled water. After a zero reading has been taken, a negative pressure of about 200 mm. (H) is established and the time t in seconds required to move the kerosene drop a given distance L is observed. Using another sample of the same membrane (s mm. square), the thickness l is measured with a micrometer and the water content W is determined by weighing before and after drying. All measurements are in millimeters and the weight is in milligrams.

The pore diameter (in millimicrons) is given by the equation

$$d = \left(56{,}600 \frac{d_c}{d_m}\right) l\ s \sqrt{\frac{L}{W\ H\ t}}$$

The ratio of the capillary diameter d_c to the effective membrane diameter d_m can be permanently determined for each apparatus by estimating the capillary diameter as follows: W' milligrams of mercury is introduced into the capillary tube and the length l_c of the column is noted. The diameter in millimeters is then equal to $d_c = 0.307 \sqrt{W'/l_c}$

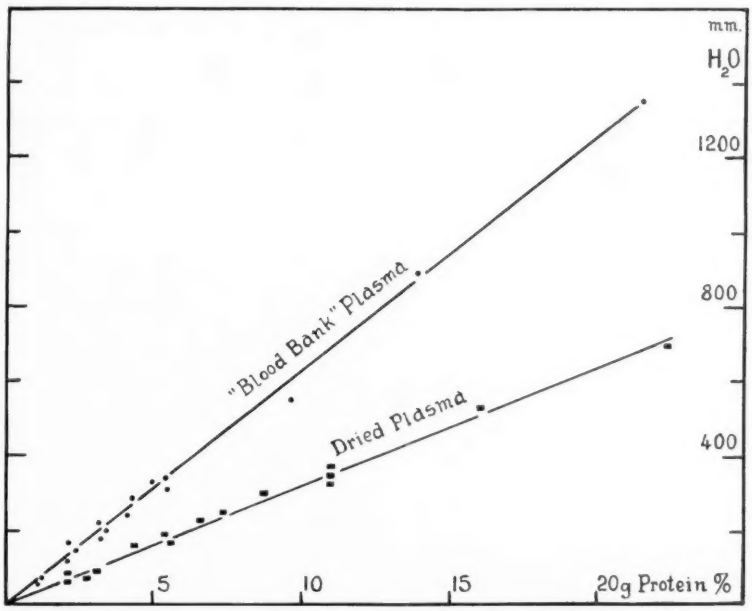

Fig. 2.—The Donnan osmotic pressures of various concentrations of human plasma, plotted against grams of protein per 100 c.c. Upper curve: Citrated human plasma from a "blood bank." The higher concentrations were obtained by dialysis in a collodion bag. Lower curve: Dried human plasma which had been found to be unsatisfactory by other tests. The various concentrations were obtained by weighing the dried powder.

In all cases the protein concentration was checked by the Barbour and Hamilton falling-drop determination of density.

Linear curves were also obtained with dilutions of horse serum, cat plasma, and a sample of approved dried human plasma.

RESULTS

Fig. 2 illustrates the results obtained with this apparatus. The curve for blood plasma at various concentrations above and below the normal level is seen to give consistent values. Another curve in Fig. 2 shows some observations on dried plasma at various concentrations. These values, although lower than for normal plasma, are consistent among themselves. Readings were found to be reproducible within 5 mm. of water.

In addition to the consistency of the readings it will be noted that throughout the range from zero up to four times normal concentration, the osmotic pressure is a linear function of the protein concentration, as measured by specific gravity. This agrees with the statement of Homer Smith and co-workers[8] and is contrary to the claim of Adair, Adair, and Greaves.[9] Further presentation of results will be included in future papers.

SUMMARY

An apparatus for measuring the "protein osmotic" or "oncotic," pressure of serum or other solutions is described. This apparatus requires only one or 2 ml. of fluid; it involves no correction for capillarity and it gives, in fifteen minutes, values which are reproducible within 5 mm. of water.

Rapid equilibrium is produced by exerting a negative pressure on the "outer" or "reference fluid" by means of a leveling bulb. When the equilibrium pressure is found, a drop of kerosene in a horizontal capillary tube becomes stationary. The Donnan osmotic pressure is then read from a manometer.

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THE MICRODETERMINATION OF GOLD IN BIOLOGICAL TISSUES*

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IN ORDER to determine small quantities of gold in tissues it was found necessary to modify the procedure described by Block and Buchanan[1] for the microdetermination of gold in blood and urine. The modifications were primarily in the digestion of the tissues, but the final procedure so differed from the original method in details that it is thought advisable to report the complete procedure with typical recoveries for tissues.

PROCEDURE

Digestion.—The tissue (0.5 to 10 Gm.) to be analyzed is placed in an accurately calibrated 100 c.c. Kjeldahl flask whose neck has been shortened to 5 or 6 cm.[1] Ten to 20 c.c. of concentrated nitric acid and a few drops of caprylic alcohol are added and the tissue is allowed to stand for a few hours until solu-

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tion has occurred. Ten to 15 c.c. of concentrated sulfuric acid are introduced into the flask which then is heated on a Kjeldahl digestion rack until the tissue is thoroughly charred. After the digest is cooled, a 30 per cent solution of hydrogen peroxide (Superoxol) is added drop by drop until the solution is water clear. The solution is evaporated to dryness over a microburner. The sulfuric acid fumes are removed by inserting into the neck of the flask a glass tube which is connected through a water trap to a water pump.[1] The contents of the flask are then treated with aqua regia to convert the gold to the chloride. If the tissues contain amounts of gold less than 200 micrograms, the color is developed and read as previously described[1] directly with the entire contents of the digestion flask. However, if larger amounts of gold are present, the aqua regia solution in the flask is heated to boiling, cooled, and transferred to a 100 c.c. volumetric flask. The Kjeldahl flask is rinsed twice with 15 to 20 c.c. of boiling aqua regia, the washings are added to the volumetric flask, which is made to volume with distilled water. Aliquots containing 50 to 150 micrograms of gold are transferred to the shortened graduated 100 c.c. Kjeldahl flasks and evaporated to dryness. Remaining traces of sulfuric acid are removed as before and the gold is reconverted to auric chloride by boiling with aqua regia. The aqua regia solution of this aliquot is then used to develop the color. Any fatty or muscular tissue (legs, gastrointestinal tract) is treated in the same fashion, except that the digestion is carried out in a 300 c.c. Kjeldahl flask with the addition of selenium as a catalyst. After evaporation to dryness with complete removal of the sulfuric acid, the residue is put into solution by boiling with three 40 c.c. portions of aqua regia. All the washings are collected in a 250 c.c. volumetric flask, made to volume with water, and aliquots containing 50 to 150 micrograms of gold are transferred to the special 100 c.c. Kjeldahl flasks. The procedure is then carried out as already described for other tissues.

This same procedure can be applied to large organs or carcasses of small experimental animals as well as to dried animal and human feces, the only modification being that a 500 c.c. Kjeldahl digestion flask is used.

Development of Color.—This is accomplished[1] by the addition of 75 c.c. of distilled water to the precipitate in the 100 c.c. Kjeldahl flask in order to dissolve the inorganic salts. Three-fourths cubic centimeter of hydrochloric acid (1:4), 8 c.c. of potassium fluoride (10 per cent), and 1 c.c. of o-dianisidine solution (0.1 per cent) are then added to the flask. The volume is made up to 100 c.c. with distilled water, the flask is stoppered and the solution thoroughly mixed. The solution is then transferred to a colorimeter absorption tube, filling the tube to within 1 to 2 cm. of the top. After stoppering, the pink color, which reaches its maximum intensity within three to ten minutes, is read in the Evelyn photoelectric colorimeter with filter 440 and a center setting of 76.

Special attention should be called to the amount of 1:4 hydrochloric acid added in the color reaction, for the rate of color development and fading depends upon the ratio of fluoride to acid. The amount of acid which remains in the flasks after conversion with aqua regia depends upon the amount of inorganic salts present. A maximum color will develop in six to eight minutes if 0.75 c.c. of 1:4 hydrochloric acid is used in direct determination of gold in tissues or urine. In determinations of gold in carcasses or feces 1.25 c.c. of the

acid must be added, and in the case of diluted tissues, 2.5 c.c. It is best to read the color at one-minute intervals during the development until the maximum color is reached and fading begins. Since it requires only fifteen seconds to make a reading, four tubes may be easily read at minute intervals.

TABLE I

RECOVERY OF KNOWN AMOUNTS OF GOLD ADDED TO TISSUES AND EXCRETA OF THE WHITE RAT

	GOLD ADDED	GOLD FOUND			GOLD ADDED	GOLD FOUND	
	mg.	mg.	per cent		mg.	mg.	per cent
Heart	0.010	0.00895	89.5	Whole	0.500	0.4683	93.7
	0.020	0.01946	97.3	legs	5.000	4.797	95.9
	0.030	0.02968	98.9		20.000	18.864	94.3
Lung	0.010	0.00928	92.8	Entire	2.000	1.829	91.5
	0.020	0.01973	98.7	carcass	10.000	9.870	98.7
	0.150	0.1471	98.1		40.000	39.420	98.5
Spleen	0.020	0.0199	99.5	Urine*	0.300	0.294	98.0
	0.100	0.100	100		5.000	4.813	96.2
	0.200	0.1916	95.8		10.000	10.000	100
Liver	0.200	0.199	99.0	Feces*	0.500	0.4064	81.3
	1.000	0.971	97.1		2.000	1.987	99.3
	5.000	4.855	97.1		5.000	4.920	98.4
Kidney	0.100	0.958	95.8				
	2.000	1.974	98.7				
	5.000	4.855	97.1				

*Gold added to urine and feces collected over a two-week period.

RESULTS

Table I gives the percentage recovery of various amounts of gold added to tissues and excreta of the white rat. The recoveries vary from 90 to 100 per cent, except in one case, in which 0.5 mg. of gold was added to feces. In this instance recovery was 81.3 per cent. This comparatively low recovery is probably associated with the inhibition of color development due to the presence of large amounts of inorganic salts in relation to the small quantity of added gold.

CONCLUSION

The method of Block and Buchanan for the microdetermination of gold in urine and blood has been modified to apply to tissues and feces. Recoveries of added gold by this procedure vary from 90 to 100 per cent.

REFERENCE

1. Block, W. D., and Buchanan, O. H.: The Microdetermination of Gold in Biological Fluids, J. Biol. Chem. **136**: 379, 1940.

A STORAGE VESSEL FOR BLOOD*

FREDERIC E. HOLMES, CINCINNATI, OHIO

THE slow rate of diffusion of gases into oil delays, but does not entirely prevent, measurable changes in the gas content of specimens stored under oil. The vessel shown in Fig. 1 was used for the anaerobic collection and storage of 10 to 15 ml. specimens of blood "under mercury." It is compact, portable, and stands firmly on its own base. It has proved satisfactory in about eight months' use in this laboratory.

In preparation of the vessel for use, the lower expanded part of the outer bulb, the storage chamber (inner bulb), and the capillary and cup above it are filled with mercury. A piece of $\frac{1}{8}$ by $\frac{3}{32}$ inch rubber tubing (not shown) is attached to the nipple in the cup and extends about 2 cm. above its rim. Liquid anticoagulant in a graduated 1 ml. pipette inserted in the top of this tube is allowed to flow toward the outer bulb until it passes through the stopcock. Then the stopcock is turned and the desired amount (usually 0.3 ml.) is delivered into the chamber. The rubber tube is removed and the cock is reopened to allow mercury to fill the capillary between cup and chamber. The rubber tube is again attached and filled with mercury from a capillary pipette or syringe.

The blood specimen is taken into a syringe containing oil. The glass tip of the syringe is inserted into the rubber tube. Any minute bubble trapped at this point is rejected through the capillary leading into the outer bulb. The unexposed specimen is allowed to flow into the chamber. When the rubber tube is removed, mercury in the cup immediately seals the top of the capillary. The cock is opened momentarily to allow mercury to flush blood from the capillary into the chamber. Mercury is swirled around the walls of the chamber by gently shaking the vessel in a slanted position in order to mix the blood with anticoagulant, and later to maintain a uniform distribution of red blood cells in the specimen during sampling. To withdraw a sample for analysis, a clean piece of rubber tubing is attached as before. Mercury from the capillary and a drop or two of blood are sucked into any convenient pipette and discarded. The sample is then drawn into a measuring pipette, such as the Van Slyke-Ostwald. The remaining unused specimen is protected by resealing with mercury in the usual manner.

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The dimensions of parts of the apparatus, which are not critical, can be obtained by comparison with the scale included in Fig. 1. For best results the capillary and bore of the plug of the cock must be free of offsets, bulges, or

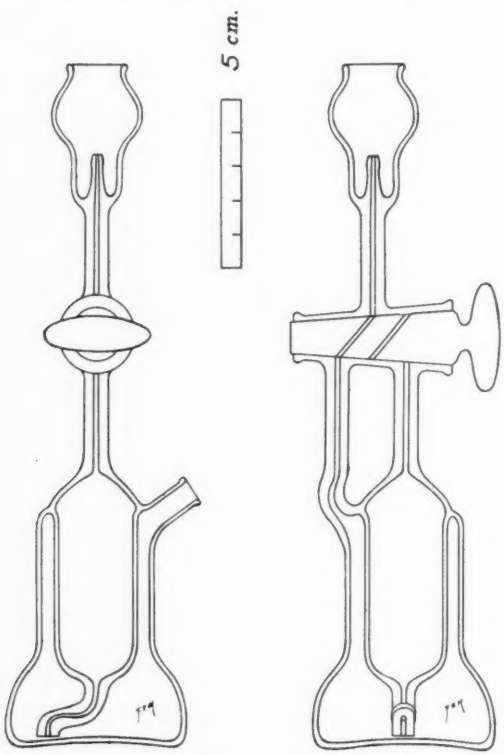

Fig. 1.—Two views of the mercury-sealed blood storage vessel. Left, from the right side. Right, front view.

pockets in which blood can collect, and should be of approximately 0.8 mm. inside diameter. The inside junction of the capillary with the top of the chamber should be abrupt so that the capillary remains filled with mercury during stirring of the specimen.

In Memoriam

ARTHUR H. THOMAS

Few names have been more closely identified with medical laboratories than has that of Arthur H. Thomas whose death occurred on August 31, 1942.

The Thomas catalogue stands on the shelves of nearly all clinical laboratories. It is doubtful whether there is a laboratory in the country which does not have many pieces of diagnostic apparatus manufactured by the Thomas Company. The name Arthur H. Thomas has appeared in the advertising pages of every issue of *The Journal of Laboratory and Clinical Medicine* from Volume 1, Number 1.

A native of Pennsylvania, Mr. Thomas entered the employ of the old Philadelphia firm of James W. Queen and Company, dealers and makers of optical and scientific instruments in 1892. He organized his own company in 1900. Since then, Arthur H. Thomas Company, dealers in laboratory apparatus and reagents, have remained among the leaders in their field.

Mr. Thomas was a Trustee and Director of Bryn Mawr College and a member of the Board of Managers of Haverford College. He was a member of the Board of the Provident Mutual Life Insurance Company.

DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TRYPSIN, Method of Assaying Suitable, for Routine Use in Diagnosis of Congenital Pancreatic Deficiency, Andersen, D. H., and Early, M. V. Am. J. Dis. Child. 63: 891, 1942.

The modified Fermi method is carried out as follows:

The substrate is a 7.5 per cent solution of Knox gelatin, obtained from the local grocer. In a 50 c.c. beaker are placed 18.75 Gm. of gelatin. About 40 c.c. of cold distilled water are gradually added, and the mixture is stirred until it is a semiliquid paste free from lumps. This paste is transferred through a small funnel to a 250 c.c. volumetric flask, and the beaker and funnel are washed with about 100 c.c. of water. The flask is heated on a water bath, with occasional shaking until the solution is clear. It is then cooled, made up to volume with water, and replaced on the water bath until the gelatin is entirely melted. The flask is shaken until the contents appear homogeneous. The gelatin is transferred to a stock bottle, and a small amount of thymol is added. When stored in the refrigerator, the substrate can be used for at least six weeks.

The substrate should be left to melt in an incubator at a temperature of 37° C. for approximately one hour before using. Twenty Wassermann tubes are set in a rack in pairs and labeled from 1 to 10; 2 c.c. of melted substrate are placed in each with a transfer pipette. One cubic centimeter of duodenal juice is placed in a 10 c.c. volumetric flask and made up to volume with a 5 per cent solution of sodium bicarbonate, this giving a 1:10 dilution. One cubic centimeter of this material is placed in a second 10 c.c. flask and made up to volume in the same way, with a resulting 1:100 dilution. Each of the first pair of tubes then receives 0.125 c.c. of the 1:100 dilution, measured with a 1 c.c. Mohr graduated pipette, and 1.875 c.c. of 5 per cent sodium bicarbonate solution; this makes a total volume of 4 c.c. in each tube, of which 0.00125 c.c. is duodenal juice. The remaining tubes are set up in a similar manner; the second pair each receives 0.25 c.c. of 1:100 dilution, and the following ones 0.5 and 1.0 c.c. of the 1:100 dilution; then 0.2, 0.4, 0.8, and 1.6 c.c. of the 1:10 dilution; and finally 0.32 c.c. of the undiluted juice in a total volume of 4 c.c. The remaining pair of tubes contains no juice and serves as a control. Each of the tubes is then covered by the thumb and inverted several times to insure mixing, care being taken to handle the control tubes first, then the pair with the lowest concentration of juice, and so on in order. The rack of tubes is incubated for one hour at 37° C. and is then left in a refrigerator at about 4° C. Twenty to twenty-four hours later the tubes are removed from the refrigerator and read immediately. The lowest dilution in which the gelatin has liquefied in one or both tubes is considered as the reading.

PNEUMONIA, Recognition of Virus Type, Goodrich, B. E., and Bradford, H. A. Am. J. M. Sc. 204: 163, 1942.

Virus-type pneumonia presents specific clinical features permitting diagnosis by positive findings. Diagnosis by exclusion alone is seldom necessary.

Chemotherapy with the sulfonamide group of drugs is not indicated in this disease. Recognition of this disease may avoid needless and possibly dangerous therapeutic efforts.

The initial roentgenographic appearance may closely simulate acute exudative tuberculosis. Progress roentgenographic study may be necessary for differentiation.

There is evidence that this disease is now showing an increasing incidence. This fact is explained not alone by familiarity with the clinical concept.

TUBERCLE BACILLUS, A New Method of Staining, Weiss, E. Am. Rev. Tuberc. 42: 199, 1942.

1. Mordant: The mordant consists of two separate solutions. Solution A contains 100 Gm. of tannic acid dissolved in 100 c.c. of 95 per cent alcohol. Solution B contains 15 c.c. of glacial acetic acid diluted to 100 c.c. with 15 per cent dilution of "solution formaldehyde" (Merck). Before using the mordant, one part of solution A and two parts of solution B are mixed together. This mixture does not require filtering and keeps for several weeks. Solutions A and B keep indefinitely.

2. Safranine (or pyronine) stain: Two and one-half grams of safranine or pyronine are dissolved in 100 c.c. of 10 per cent alcohol. The dissolving process is facilitated by the application of heat. The solutions are then filtered and keep indefinitely.

3. Decolorizer: Fifteen cubic centimeter of glacial acetic acid are diluted to 100 c.c. with acetone. This decolorizer keeps very well.

4. Löffler's methylene blue: Thirty cubic centimeters of a saturated alcoholic solution of methylene blue (5 Gm. dissolved in 100 c.c. of 95 per cent alcohol) are diluted with 100 c.c. of a 1:10,000 solution of potassium hydroxide. The filtrate keeps well.

Staining Technique.—(1) Prepare uniformly thin smears and fix the dry smears over the flame. (2) Cover the slides with the foregoing mordant mixture and steam for five minutes. (3) Wash with running water. (4) Cover the smears with the above safranine or pyronine stain and steam for five minutes. (5) Wash with running water. (6) Decolorize the slides with the above decolorizer until they become colorless. (7) Wash vigorously with running warm water. (8) Cover the smears with Löffler's methylene blue for one minute. (9) Wash with running water and allow the slides to dry. The tubercle bacilli appear as large, thick, deeply red-stained rods while the other bacteria and tissue structures are stained blue.

Evaluation of the procedure: The proposed method has been compared with the Ziehl-Neelsen method on at least 250 diagnostic smears. The tubercle bacilli appeared not only thicker and larger but also more numerous. These observations are based on actual counts performed on the above specimens.

ALKAPTONURIA, The Instantaneous Diagnosis of, on a Single Drop of Urine, Fishberg, E. H. J. A. M. A. **119:** 882, 1942.

The urine is made strongly alkaline with sodium or potassium hydroxide and dropped on regulation sensitized photographic paper. Wherever a drop of the urine touches the paper, the latter turns coal black instantly, a process similar to that used in everyday photography. This may be done in full daylight.

No other substance present in urine, either normally or pathologically, is capable of reducing the photographic paper in this manner. Diabetic urine, no matter how high the concentration of reducing sugar, remains without effect. Also, the higher concentrations of vitamin C present in urine after metabolic experiments do not act in this manner.

Since alkaptonuria is either total or absent and the substance is excreted in quantities as high as 16 Gm. a day, usually, however, about 3 to 5 Gm., the reaction is unlikely to be negative in an alkaptonuric patient at any time.

ORAL GLUCOSE TOLERANCE TESTS, Instances of Disagreement in the Results of Two Types of, Langner, P. H., and Dewees, E. J. Am. J. M. Sc. **204:** 85, 1942.

From a group of 160 subjects who had submitted to the two-dose, one-hour glucose tolerance test, 16 cases are presented in which the results were border line or slightly abnormal. A one-dose test was also performed on these persons. In 13 instances the one-dose test and the available clinical data indicated a normal carbohydrate metabolism and, therefore, were in disagreement with the two-dose glucose tolerance test. The authors believe that the conventional one-dose glucose tolerance test is more reliable than the newer Exton-Rose procedure.

PORTAL CIRRHOSIS, Gottardo, Paul, and Winters, W. L. Am. J. M. Sc. 204: 205, 1942.

A series of 24 cases of portal cirrhosis of the liver in varying stages was studied in an attempt to correlate clinical and laboratory findings. The diagnosis was confirmed by peritoneoscopy in 20 cases and by post-mortem examination in 4 cases. Marked change in the serum albumin and globulin were noted in 100 per cent of the cases. There was a marked tendency to the lowering of the serum albumin and a rise of the serum globulin, with a consequent lowering of the albumin globulin ratio in all cases. There was a reversal of the albumin-globulin ratio in two-thirds of the cases. Jaundice was a fairly constant feature, being present in some degree, as measured by the icteric index, in all cases. Jaundice was evident on physical examination in 79 per cent of the cases. Changes in the peripheral blood were marked in 96 per cent of the cases. A macrocytic or hyperchromic anemia was present in 67 per cent of the cases while 25 per cent exhibited a microcytic or hypochromic anemia. One case presented a typical severe sickle-cell anemia. Deficiency of liver function, as determined mainly by the hippuric acid test and also by a glucose tolerance and a bromsulfalein test, was noted in 83 per cent of the cases. When the serum protein determinations were utilized as a liver function test, 100 per cent of the cases exhibited a diminution of liver function.

A correlation of clinical and laboratory findings, together with peritoneoscopic examination of the liver and post-mortem analysis, would appear to offer useful fields of investigation in liver disease.

RENAL GLYCOSURIA, The Renal Blood Flow, Glomerular Filtration Rate and Degree of Tubular Reabsorption of Glucose in, Friedman, M., Selzer, A., Sugarman, J., and Sokolow, M. Am. J. M. Sc. 204: 22, 1942.

The effective renal blood flow (diodrast clearance) and the rate of glomerular filtration and the degree of renal tubular reabsorption of glucose were studied in 7 normal persons, and in 5 patients with renal glycosuria.

The effective renal blood flow and glomerular filtration in the patient with renal glycosuria were found to be normal.

The lessened tubular reabsorption of glucose in the person with renal glycosuria does not appear to be due to an organic kidney defect, for at plasma glucose levels above 200 mg. per 100 c.c. the efficiency of tubular reabsorption of glucose in these cases equals or exceeds that found in nonglycosuric patients.

GALACTOSE TOLERANCE, Studies on, With Special Reference to Thyroid Disease, Rosenkrantz, J. A., Bruger, M., and Lockhart, A. J. Am. J. M. Sc. 204: 35, 1942.

The factor of age per se appears to exert little or no influence on the galactose tolerance curve.

Patients with hyperthyroidism, Bright's disease, upper respiratory infections, malignant disease, and those who have recently received sulfonamide therapy frequently demonstrate impairment in galactose tolerance following the oral administration of this sugar. The impairment in galactose tolerance, however, is most marked in patients with thyrotoxicosis, which may be ascribed to a dual mechanism: (a) increased rate of absorption of this sugar from the intestinal tract and (b) hepatic damage.

Patients with diabetes mellitus are shown to have a normal tolerance for ingested galactose.

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